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The molecular genetic basis of prostate cancer is poorly understood. We originally proposed to identify the nes that are differentially expressed between normal prostate tissue and prostate cancer. Tumor suppressor genes e one type of such genes. In the funding period, we focused on mapping, identifying, and characterizing tumor ppressor genes in prostate cancer. We applied the methods of tissue microdissection and deletion mapping to calize tumor suppressor genes on human chromosome 13. We also used PCR-SSCP and direct sequencing methods analyze candidate genes for their involvement in prostate cancer. We identified three regions of chromosome 13 r harboring tumor suppressor gene, i.e., 13q14, 13q21, and 13q33. The ERCC5/XPG DNA repair gene at 13q33 as not involved in prostate cancer, neither was the RB1 gene at 13q14 and the EDNRB gene at 13q21. Therefore, ch of the three tumor suppressor genes remains to be identified. More interestingly, alterations of 13q14 and 13q21 peared to be specifically responsible for the aggressive behavior of prostate cancer. We further defined the region deletion, at 13q21, and found that some human tumors have homozygous deletion at 13q21. We have constructed physical map for 13q21 deletion region. We also used polymerase chain reaction (PCR)-single strand nformational polymorphism (SSCP) and direct sequencing methods to examine mutations of PTEN in high grade imary tumors and metastases of prostate cancer. We found that PTEN mutation is significantly more frequent in gh grade prostate cancers, indicating that PTEN is more likely involved in the progression of prostate cancer. Based the data generated in this project, we were awarded two NIH R01 grants from the National Cancer Institute.

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INTRODUCTION:

The molecular genetic basis of prostate cancer is poorly understood, that hampers optimal diagnosis, prognosis, and therapy of patients with prostate cancer. We originally proposed to identify the genes that are differentially expressed between normal prostate tissue and prostate cancer. Many of such genes are believed to be nonspecific events accompanying the cancer phenotype. Only a small portion of them play a causal role in cancer development. During the funding period, we focused on mapping, identifying, and characterizing tumor suppressor genes in prostate cancer. We collected tumor cells and normal cells from a large number of specimens of prostate cancer. We then examined the genomes of these materials for chromosomal and genetic abnormalities using PCR-based methods. Completion of this project has led to the publication of six papers in biomedical journals and the award of two NIH R01 grants from the National Cancer Institute.

BODY:

In this project, we performed a series of experiments to identify genes that are involved in the development and progression of prostate cancer, as originally planned. We targeted two genes. One is a novel tumor suppressor gene at the q21 band of human chromosome 13 (13q21), and the other is the PTEN tumor suppressor gene, the most frequently altered gene in human prostate cancer.

We first performed loss of heterozygosity (LOH) analysis for chromosome 13 in human prostate cancer (1). Chromosome 13 is one of the most frequently altered chromosomes in prostate cancer. Two known tumor suppressor genes, *RB1* and *BRCA2*, map to chromosome 13; however, recent reports suggest that unknown genes on 13q are more likely to be involved in the development of prostate cancer. In order more fully to define the genetic changes on chromosome 13 in prostate neoplasms, we analyzed 27 polymorphic microsatellite markers spanning the q arm for loss of heterozygosity in 40 primary tumors and in metastases from 11 other patients who died of prostate cancer. Of the 40 primary tumors, 23 (58%) showed LOH for at least one marker. Three distinct regions at q14, q21-22, and q33, defined by markers D13S267 and D13S153, D13S166 and D13S1225, and D13S259 and D13S274, showed the most frequent LOH, suggesting their involvement in the development of prostate cancer. For the 12 patients whose tumors showed LOH at these markers, the average age at diagnosis was 57 years, which was younger than that (62 years, $P < 0.05$) for the 28 patients whose tumors lacked LOH. Ten of the 11 (91%) metastases showed LOH with one or more markers. Two of the three most frequently deleted regions (i.e., q14 and q21-22) in the primary tumors and markers linked to the *RB1*, *BRCA2*, and *EDNRB* genes showed high frequencies (56-71%) of LOH in metastases. These results demonstrate that allelic loss on chromosome 13 at q14, q21-22, and q33 occurs in a subset of primary prostate tumors and is a frequent event in metastatic lesions of prostate cancer.

We then examined the frequency of 13q LOH in advanced prostate cancers, in order to determine the clinicopathologic relevance of 13q LOH (2). LOH was determined by analyzing microsatellite markers in 41 cases of microdissected predominantly high grade prostate cancer tissues and their matched nonneoplastic cells. The results were compared with those generated previously for lower grade, asymptomatic cancers (1). The

frequencies of LOH at 13q14, 13q21, and 13q33 were 62% (21/34), 57% (20/35), and 34% (11/32), respectively. In comparison to previous results, LOH at 13q14 and 13q21 but not 13q33 was more frequent in prostate cancers that produced local clinical symptoms (bladder outlet obstruction) than those that did not ($P < 0.05$). LOH at 13q14 was also significantly more frequent in high grade and high stage cancers than those that were lower grade and lower stage ($P < 0.05$). These findings suggest that LOH at 13q14 in particular is associated with clinically significant prostate cancers.

To better define the region of deletion at 13q21 for the eventual cloning of the 13q21 tumor suppressor gene, we examined prostate cancer specimens and cell lines/xenograft for hemizygous and homozygous deletions at 13q21, using the methods of tissue microdissection and duplex PCR (3). Deletions at 13q21 were detected in 13 of 147 (9%) prostate cancer samples. Deletion of the same region was also detected in the LNCaP cell line and the PC-82 xenograft of prostate cancer. The overlapping region of deletion in LNCaP and PC-82 spans 3.1 cM or 2.9 cR, which is equivalent to 1-3 Mb. The endothelin receptor B gene, a possible tumor suppressor gene at 13q21, was not located in the region of deletion. Among the 13 prostate neoplasms with deletion at 13q21, 5 were metastases, and 7 were poorly differentiated primary tumors. The only primary tumor that was not poorly differentiated but had deletion occurred in one of the youngest patients (49 years) at diagnosis. These results provide additional evidence that 13q21 harbors an unidentified gene(s) whose inactivation occurs in some aggressive carcinomas of the prostate.

To fine-map the area of deletion, we established a contig of bacterial artificial chromosome (BAC) clones, narrowed the region of deletion by loss of heterozygosity (LOH) and homozygosity-mapping-of-deletion (HOMOD) analyses in different types of cancers, and tested a candidate gene from the region for mutation and alteration of expression in prostate cancers (4). The contig consisted of 75 overlapping BAC clones. In addition to the generation of 47 new sequence-tagged-site (STS) markers from the ends of BAC inserts, 76 known STS and expressed sequence tag markers were mapped to the contig (25 kb per marker on average). The minimal region of deletion was further defined to be about 700 kb between markers D13S791 and D13S166 by LOH analysis of 42 cases of prostate cancer, and by HOMOD analysis of eight prostate cancer cell lines/xenografts and 49 cell lines from cancers of the breast, ovary, endometrium, and cervix, using 18 microsatellite markers encompassing the deletion region. A gene that is homologous to the WT1 tumor suppressor gene, AP-2rep (KLF12), was mapped in this region and was analyzed for its expression and genetic mutation. In addition to low levels of expression in both normal and neoplastic cells of the prostate, this gene did not have any mutations in a group of aggressive prostate cancers and cell lines/xenografts, as assessed by the methods of polymerase chain reaction-single strand conformational polymorphism analysis and direct sequencing. These studies suggest that a 700 kb interval at 13q21 harbors a tumor suppressor gene(s) that seems to be involved in multiple types of cancer, and that the AP-2rep gene is unlikely to be an important tumor suppressor gene in prostate cancer. The BAC contig and high-resolution physical map of the defined region of deletion should facilitate the cloning of a tumor suppressor gene(s) at 13q21

One of the three regions of chromosome 13 previously identified for having LOH in human prostate cancer, i.e., 13q33 defined by markers D13S158 and D13S280, harbors the XPG/ERCC5 DNA repair gene. Germline mutation of this gene leads to xeroderma pigmentosum, suggesting its candidacy for the 13q33 tumor suppressor gene. We therefore

performed LOH and mutational analysis of the XPG gene in human prostate cancers, in order to determine whether the XPG gene is involved in the development of prostate cancer (5). LOH of the XPG gene was analyzed in 40 primary prostate cancers and 14 metastases by using the microsatellite assay, and its mutations were examined in 5 cell lines, 14 metastases, and 8 tumors with LOH at 13q33 by using the single-strand conformation polymorphism (SSCP)-direct DNA sequencing analysis. Four of the 29 (14%) informative primary tumors and 4 of 8 (50%) metastases showed LOH for the XPG gene. Analysis of the 8 tumors with LOH at the 13q33 region, 14 metastases, and 5 cell lines of prostate cancer revealed two polymorphisms but no mutation of the gene. The polymorphism in exon 2 did not change the amino-acid sequence of the XPG protein, but the exon 15 polymorphism altered codon 1104 from histidine to aspartic acid. The two polymorphisms also occurred in individuals without prostate cancer. LOH at XPG in prostate cancer supports the conclusion that the 13q33 region contains a gene important in the development of prostate cancer, while lack of mutations of the gene suggests that XPG is not the target gene involved.

PTEN/MMAC1 is a putative tumor suppressor gene located on 10q23, one of the most frequently deleted chromosomal regions in human prostate cancer. While mutations of *PTEN* have been often detected in metastases of prostate cancer, localized tumors have shown lower rates of mutation, which have varied from zero to 20% among different studies. It is unknown whether the rate of *PTEN* mutations is different in prostate cancer from Asian compared with Western men. To further clarify the role of *PTEN* in prostate cancer and to examine the gene for mutations in Asian men, we analyzed 32 cases of primary prostate cancers from Chinese patients, each of whom was not diagnosed by screening with serum PSA, for *PTEN* mutations using the methods of tissue microdissection, single strand conformation polymorphism, and direct DNA sequencing (6). Seventy percent of the tumors were Gleason score 8-10, while the remainder were Gleason score 7. Six metastases of prostate cancer from American patients were also analyzed. Five of 32 (16%) primary prostate cancers from Chinese men and two of six metastases from American men showed mutations in a total of 10 codons of *PTEN*, which involved exons 1, 2, 5, 8, and 9. Two of the mutations were truncation type, while the rest were missense mutations. The mutation frequency in these cases from Asian patients was higher than that in our previous study of cases in radical prostatectomy specimens from American men, in which the 40 primary tumors were lower grade and had been detected by serum PSA test (7). We conclude that mutation of *PTEN* occurs more often in primary prostate cancers of Chinese men, whose tumors are high grade and reflective of an unscreened population.

The above studies have been published in the following six papers:

Chen, C., Brabham, W. W., Stultz, B. G., Frierson, H. F. J., Barrett, J. C., Sawyers, C. L., Isaacs, J. T., and Dong, J. T. (2001). Defining a common region of deletion at 13q21 in human cancers. Genes Chromosomes Cancer *31*, 333–344.

Dong, J. T., Boyd, J. C., and Frierson, H. F., Jr. (2001a). Loss of heterozygosity at 13q14 and 13q21 in high grade, high stage prostate cancer. Prostate *49*, 166-171.

Dong, J. T., Chen, C., Stultz, B. G., Isaacs, J. T., and Frierson, H. F., Jr. (2000). Deletion at 13q21 is associated with aggressive prostate cancers. Cancer Res *60*, 3880-3883.

Dong, J. T., Li, C. L., Sipe, T. W., and Frierson, H. F. J. (2001b). Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients. Clin Cancer Res *7*, 304-308.

Hyytinen, E. R., Frierson, H. F., Boyd, J. C., Chung, L. W. K., and Dong, J. T. (1999a). Three distinct regions of allelic loss at 13q14, 13q21-22, and 13q33 in prostate cancer. Genes Chromosomes Cancer *25*, 108-114.

Hyytinen, E. R., Frierson, H. F., Sipe, T. W., Li, C. L., Degeorges, A., Sikes, R. A., Chung, L. W. K., and Dong, J. T. (1999b). Loss of heterozygosity and lack of mutations of the XPG/ERCC5 DNA repair gene at 13q33 in prostate cancer. Prostate *41*, 190-195.

These papers are included in the appendix.

KEY RESEARCH ACCOMPLISHMENTS:

- Chromosome 13 has three distinct regions of LOH at 13q14, 13q21, and 13q33 in prostate cancer.
- The ERCC5 DNA repair gene at 13q33 is not the target gene in prostate cancer.
- The RB1 tumor suppressor gene is not the target gene at 13q14.
- The EDNRB gene, a tumor suppressor gene located at 13q21, is not the target gene of 13q21 deletion.
- Homozygous deletion occurs at 13q21 in prostate cancer.
- A physical map for the region of 13q21 deletion has been constructed.
- The genes at 13q14 and 13q21 are currently unknown and need to be identified.
- Alterations at 13q14 and 13q21 are responsible for the aggressive behavior of prostate cancer.
- Mutations of the PTEN gene occurs more frequently in high grade tumors and metastases of prostate cancer. PTEN thus plays a role in the progression of this disease.

REPORTABLE OUTCOMES:

Completion of this project has generated six papers and two NIH R01 grants funded by the National Cancer Institute. The six papers are:

1. Hyytinen ER, Frierson HF, Boyd JC, Chung LWK, and Dong JT. Three distinct regions of allelic loss at 13q14, 13q21-22, and 13q33 in prostate cancer. Genes Chromosomes Cancer *25:* 108-114, 1999.
2. Dong JT, Boyd JC, and Frierson HF, Jr. Loss of heterozygosity at 13q14 and 13q21 in high grade, high stage prostate cancer. Prostate *49:* 166-171, 2001.
3. Dong JT, Chen C, Stultz BG, Isaacs JT, and Frierson HF, Jr. Deletion at 13q21 is associated with aggressive prostate cancers. Cancer Res *60:* 3880-3883, 2000.
4. Chen C, Brabham WW, Stultz BG, Frierson HFJ, Barrett JC, Sawyers CL, Isaacs JT, and Dong JT. Defining a common region of deletion at 13q21 in human cancers. Genes Chromosomes Cancer *31:* 333–344, 2001.

5. Hyytinen ER, Frierson HF, Sipe TW, Li CL, Degeorges A, Sikes RA, Chung LWK, and Dong JT. Loss of heterozygosity and lack of mutations of the XPG/ERCC5 DNA repair gene at 13q33 in prostate cancer. Prostate *41:* 190-195, 1999.
6. Dong JT, Li CL, Sipe TW, and Frierson HFJ. Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients. Clin Cancer Res *7:* 304-308, 2001.

The two R01 grants are:

(1). Period: 4/1/00 - 3/31/05; Title: *Molecular dissection of 13q14 in prostate cancer.* Agency: NIH/NCI; P.I.: Dong JT; Award Number: R01 CA 85560.

(2). Period: 8/18/00 - 7/31/05; Title: *A tumor suppressor gene at 13q21 in prostate cancer.* Agency: NIH /NCI; P.I.: Dong JT; Award Number: R01 CA 87921.

CONCLUSIONS:

In this project, we have localized three tumor suppressor genes at 13q14, 13q21, and 13q33 in prostate cancer. The loci at 13q14 and 13q21 appeared to be specifically involved in aggressive prostate cancer, that is particularly important because currently it is more urgent to find molecular markers that can differentiate clinically aggressive prostate cancers from those latent ones. We also fine mapped the region of deletion at 13q21, and excluded several known genes as the target genes of deletion. We also clarified the role of the PTEN tumor suppressor gene in prostate cancer. PTEN is so far the most frequently mutated gene in prostate cancer, but we found that PTEN is specifically involved in high grade primary tumors or metastases of prostate cancer.

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1. Hyytinen ER, Frierson HF, Boyd JC, Chung LWK, and Dong JT. Three distinct regions of allelic loss at 13q14, 13q21-22, and 13q33 in prostate cancer. Genes Chromosomes Cancer *25:* 108-114, 1999.
2. Dong JT, Boyd JC, and Frierson HF, Jr. Loss of heterozygosity at 13q14 and 13q21 in high grade, high stage prostate cancer. Prostate *49:* 166-171, 2001.
3. Dong JT, Chen C, Stultz BG, Isaacs JT, and Frierson HF, Jr. Deletion at 13q21 is associated with aggressive prostate cancers. Cancer Res *60:* 3880-3883, 2000.
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5. Hyytinen ER, Frierson HF, Sipe TW, Li CL, Degeorges A, Sikes RA, Chung LWK, and Dong JT. Loss of heterozygosity and lack of mutations of the XPG/ERCC5 DNA repair gene at 13q33 in prostate cancer. Prostate *41:* 190-195, 1999.
6. Dong JT, Li CL, Sipe TW, and Frierson HFJ. Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients. Clin Cancer Res *7:* 304-308, 2001.
7. Dong JT, Sipe TW, Hyytinen ER, Li CL, Heise C, McClintock DE, Grant CD, Chung LW, and Frierson HF, Jr. PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate. Oncogene *17:* 1979-1982, 1998.

GENES, CHROMOSOMES & CANCER 25:108–114 (1999)

Three Distinct Regions of Allelic Loss at 13q14, 13q21-22, and 13q33 in Prostate Cancer

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Chromosome 13 is one of the most frequently altered chromosomes in cancer, including carcinoma of the prostate. Two known tumor suppressor genes, *RB1* and *BRCA2*, map to chromosome 13; however, recent reports suggest that unknown genes on 13q are more likely to be involved in the development of prostate cancer. In order more fully to define the genetic changes on chromosome 13 in prostate neoplasms, we analyzed 27 polymorphic microsatellite markers spanning the q arm for loss of heterozygosity in 40 primary tumors and in metastases from 11 other patients who died of prostate cancer. Of the 40 primary tumors, 23 (58%) showed LOH for at least one marker. Three distinct regions at q14, q21-22, and q33, defined by markers *D13S267* → *D13S153*, *D13S166* → *D13S1225*, and *D13S259* → *D13S274*, showed the most frequent LOH, suggesting their involvement in the development of prostate cancer. For the 12 patients whose tumors showed LOH at these markers, the average age at diagnosis was 58 years, which was younger than that (63 years, $P < 0.05$) for the 28 patients whose tumors lacked LOH. Ten of the 11 (91%) metastases showed LOH with one or more markers. Two of the three most frequently deleted regions (i.e., q14 and q21-22) in the primary tumors and markers linked to the *RB1*, *BRCA2*, and *EDNRB* genes showed high frequencies (56–71%) of LOH in metastases. These results demonstrate that allelic loss on chromosome 13 at q14, q21-22, and q33 occurs in a subset of primary prostate tumors and is a frequent event in metastatic lesions of prostate cancer. *Genes Chromosomes Cancer 25:108–114, 1999.* © 1999 Wiley-Liss, Inc.

INTRODUCTION

The development and subsequent progression of prostate cancer are a complex process requiring multiple genetic abnormalities. An overview of these genetic aberrations has been obtained by comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analysis (Kunimi et al., 1991; Cher et al., 1994, 1996; Visakorpi et al., 1995; Cunningham et al., 1996). Losses on 6q, 8p, 10q, 13q, 16q, 17p, and 18q are consistently detected, thus providing targets for further studies aimed at identifying the genes residing in these regions. CGH studies have indicated that chromosome 13 is the second most commonly altered chromosome (after chromosome 8) in prostate cancer, showing loss of the q arm in 32% of primary tumors and in 56–75% of recurrent and metastatic tumors. Moreover, each of the four cell lines derived from metastatic prostate cancers, i.e., LNCaP, DU-145, PC-3, and TSU-Pr1, showed loss of 13q sequence with the minimal common deleted region at 13q21 (Nupponen et al., 1998). In addition, loss of chromosome 13 occurs in a number of other cancers such as those of the lung, breast, ovary, and kidney (Yang-Feng et al., 1993; Kuroki et al., 1995; Schoenberg et al., 1995; Maestro et al., 1996; Forozan et al., 1997; Kalachikov et al., 1997; Tamura et al., 1997).

Chromosome 13 harbors the tumor suppressor genes (TSGs) *RB1* and *BRCA2*, which are located at the 13q14.2 and 13q12.2 chromosomal regions, respectively. Allelic loss at the *RB1* locus has been found in about one third of clinically localized prostate tumors (Brooks et al., 1995; Melamed et al., 1997), and mutations of the gene have been detected in a few primary prostate cancers (Kubota et al., 1995). No correlation between LOH and mutation or absence of expression of the *RB1* gene has been observed, however (Cooney et al., 1996; Ittmann and Wieczorek, 1996; Latil et al., 1996). The locus with the most frequent LOH in the q14 region has been found to lie in a 7-cM interval encompassing the loci of *D13S263* and *RB1* (*D13S153*) (Cooney et al., 1996; Ittmann and Wieczorek, 1996; Latil et al., 1996). The *BRCA2* gene has not been found to be frequently altered in primary prostatic neoplasms (Cooney et al., 1996; Ittmann and Wieczorek, 1996; Latil et al., 1996; Melamed et al., 1997).

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The results of CGH and LOH studies suggest that unidentified genetic loci on 13q are involved more frequently in the development of prostate cancer. No detailed deletion maps using multiple markers that span the entire 13q have been constructed, however. We performed LOH analysis using 27 polymorphic microsatellite markers spanning the q arm of chromosome 13 in 40 primary prostate cancers and in metastases from 11 other patients who died of prostate cancer. Three regions defined by the markers *D13S267* → *D13S153*, *D13S166* → *D13S1225*, and *D13S259* → *D13S274* at q14, q21-22, and q33, respectively, were identified as having the most frequent LOH in a subset of prostate cancers; two of these LOH regions were lost even more frequently in prostate cancer metastases.

MATERIALS AND METHODS

Tumor Samples

Forty pairs of matched zinc formalin-fixed, paraffin-embedded normal and primary prostate cancer tissues from prostatectomy specimens from previously untreated patients were used in this study. Patient ages ranged from 42 to 74 years (median, 60 years). The pathologic characteristics of the tumors were as follows: Gleason score: 82% had scores of 5 to 7, whereas 18% had scores of 8 or 9; lymph node metastases: one of 40 cases; seminal vesicle invasion: 83% negative and 17% positive; capsule penetration: 64% negative and 36% positive. The tumors were staged pathologically according to the most recent AJCC recommendations (AJCC, 1997). Two thirds were pT2 and one third were pT3 cancers. In addition, 11 metastatic cancer specimens from lymph nodes, liver, or bone and matched non-neoplastic tissues from patients who had died of prostate cancer were obtained at autopsy. Tumors were zinc formalin-fixed and paraffin-embedded, and the cells for DNA isolation were collected from 7-μm H- and E-stained sections using a previously described protocol for preparation of histologic sections on glass slides prior to microdissection (Moskaluk and Kern, 1997). Tumor samples were microdissected to ensure a minimum number of 70% neoplastic cells. Non-neoplastic cells from lymph nodes or seminal vesicles (or spleen for autopsy specimens) in almost 90% of the cases, and from normal prostate in the remainder, were obtained from paraffin blocks that contained no neoplastic cells.

LOH Analysis

DNA was isolated from specimens by adding proteinase K solution, incubating at 55°C for 2–3 days, extracting with phenol and chloroform, and precipitating with ethanol. Twenty-seven microsatellite markers, which were either purchased from Research Genetics (Huntsville, AL) or synthesized by Life Technologies (Rockville, MD), were used. The chromosomal location of these markers and their genetic distances (cM) from the top of chromosome 13 have been established as follows (Hudson et al., 1995): *D13S217* (q12.1, 19), *D13S267* (q12.3, 28.9), *D13S263* (q14.1-14.2, 40), *D13S1227* (q14.1-14.2, 41.7), *D13S153* (q14.2, 47.5), *D13S119* (q14.3-21, 51.5), *D13S312* (q21.1-21.3, N/A), *D13S134* (q21.2-22, N/A), *D13S166* (q21.3-22, 57.3), *D13S156* (q21.3-22, 57.3), *D13S313* (q21.1-21.3, N/A), *D13S269* (q21.3-22, 58), *D13S162* (q22, 60), *D13S1306* (q22, 61), *D13S1225* (q22, 64.3), *D13S264* (q22, 65.5), *D13S317* (q22, 66), *D13S154* (q31, 77.1), *D13S121* (q31, N/A), *D13S159* (q32, 81.5), *D13S1240* (q32, 83.7), *D13S259* (q33, 86.9), *D13S158* (q33, 86.9), *D13S280* (q33, 87.5), *D13S274* (N/A, 89.4), *D13S173* (q33-34, 96), and *D13S285* (q34, 113). Three of the markers were physically mapped either at or near known genes. *D13S267* is linked to the *BRCA2* gene (Wooster et al., 1995), *D13S153* is from intron 2 of the *RB1* gene (Toguchida et al., 1993), and *D13S317* is located in a YAC clone that harbors the *EDNRB* gene (Puffenberger et al., 1994).

For the 11 prostate cancer metastases, 14 markers that had LOH at different frequencies (most with high rates, some with lower rates) in informative localized tumors were analyzed in addition to the markers linked to the known gene loci. The sequences of PCR primers for these markers were obtained from the Genome Database (http://gdbwww.gdb.org) and the Center for Genome Research at the Whitehead Institute for Biomedical Research (http://www-genome.wi.mit.edu). Each PCR analysis was performed on 10–25 ng of genomic DNA; 1 × PCR buffer (1.5 mM $MgCl_2$); 0.4 μM of each primer; 200 μM dCTP, dGTP, and dTTP; 5 μM dATP; 2.5 μCi of α-^{35}S-dATP (1,000 mCi/mmol); and 1 U of Taq DNA polymerase in a volume of 10 μl. Thirty-five to 40 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 48–57°C for 30 sec, and extension at 72°C for 1 min, were performed.

PCR products were separated in a 6% denaturing polyacrylamide gel and autoradiographed for 2–5 days using Kodak BioMax MR films. Allelic loss was determined when the signal for one allele in the tumor was reduced significantly when compared to that for the non-neoplastic cells. The change in the size of an allele in a cancer tissue

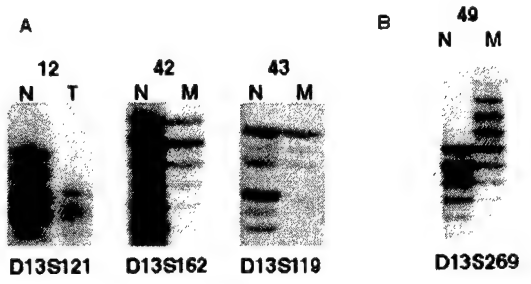

Figure 1. Examples of allelic loss (**A**) and microsatellite instability (**B**) in prostate cancer obtained with microsatellite markers of chromosome 13. N, T, and M indicate normal and matched tumor or metastatic tissue, respectively, for four cases. Case number is on the top.

specimen compared to its matched non-neoplastic tissue sample was termed microsatellite instability (MSI). Examples of microsatellite alterations, which include LOH and MSI, are shown in Figure 1 for markers on chromosome arm 13q. All autoradiograms were analyzed independently by two of the investigators (E.-R.H. and J.-T.D.), and any conflicts in interpretation were resolved by repetition of the experiment.

The relationship between LOH at loci of *D13S263-D13S1227*, *D13S313-D13S269-D13S162*, and *D13S158-D13S280* and patient age at diagnosis, Gleason score, or tumor stage was analyzed statistically by use of the Wilcoxon sign rank test.

RESULTS

Primary Tumors

Of the 40 primary tumors analyzed, 23 (58%) had LOH for at least one marker. LOH at the 27 loci for the primary tumors ranged from 0 to 27% (Fig. 2). Whereas most markers showed LOH in less than 5% of tumors, markers *D13S263*, *D13S313*, *D13S269*, and *D13S158* had allelic loss in at least 14% of informative cases. These markers defined three distinct regions. The most centromeric region (18.6 cM) was at 13q14.2 and was defined by markers *D13S267* and *D13S153*, because markers *D13S263* and *D13S1227* showed LOH in 5/33 cases (15%) and 3/28 (11%) of informative cases, respectively, and 3 tumors had LOH at both of these markers (Fig. 2). This region seems to be different from that of *RB1* (*D13S153*) at 13q14, because none of the tumors with LOH for *D13S263* and *D13S1227* showed LOH at *RB1*. In addition, *RB1* showed LOH in only 1/26 (4%) informative cases. The intermediate region (7 cM) was defined by markers *D13S166* and *D13S1225* at 13q21-22, as *D13S313*, *D13S269*, *D13S162*, and *D13S1306* together showed LOH in seven tumors. The telomeric region (2.5 cM) was at 13q33 and lay between markers *D13S259* and *D13S274*, because five tumors showed LOH at either *D13S158* or *D13S280* or both, whereas alleles were either retained or uninformative for the flanking markers *D13S259* and *D13S274* (Fig. 2). There were 12 tumors that had LOH in at least one of the markers from the three LOH regions. These tumors had a mean Gleason score of 7 and occurred in patients whose age at diagnosis averaged 58 years; 50% of them had a higher local stage (pT3) at prostatectomy. The 28 tumors without LOH at these regions also had intermediate Gleason scores and occurred in patients with an average age at diagnosis of 63 years; 32% of them occurred at stage pT3 (Table 1). The difference in age at diagnosis between these two groups was statistically significant ($P < 0.05$).

The primary cancers with the most frequent microsatellite alterations (including both LOH and MSI) were cases 6, 12, 13, 19, 21, and 25, in which alterations were detected in 6/20 (30%), 5/18 (28%), 7/15 (47%), 3/14 (21%), 7/22 (32%), and 6/19 (32%) of the informative loci respectively. Most of the microsatellite alterations for these six tumors were at the loci of *D13S263*, *D13S313*, *D13S269*, *D13S162*, and *D13S158*. Except for case 13, all the other five cases were among the 12 tumors showing LOH at the three regions most frequently lost. MSI was detected sporadically in 15 (38%) of the tumors. Only one tumor (case 13) appeared to have a replication error phenotype, because each of the allelic alterations was an MSI. The *D13S162* locus from the q22 region showed MSI in nine of the 28 informative cases (32%).

Metastatic Lesions

For the metastases from the 11 patients at autopsy, we analyzed 14 microsatellite markers that were located on different bands of chromosome 13. Ninety-one percent (10/11) of the cases showed LOH with at least one marker (Fig. 3). Markers *D13S263*, *D13S313*, and *D13S269*, which had the highest frequency of LOH in the primary tumors, were also among the markers that had high frequencies (56–71%) of LOH in the metastases. In addition, the three loci linked to the *BRCA2*, *RB1*, and *EDNRB* genes (*D13S267*, *D13S153*, and *D13S317*) were also among the loci with high rates of LOH in the metastases (71, 56, and 56%, respectively). Similar to the findings in the primary tumors, most of the alterations for marker *D13S162* in metastases were scored as MSI rather than LOH. Five of the 11 metastases (cases 42, 43, 44, 46, and 47) showed extensive (>75%) allelic alterations (4 with LOH

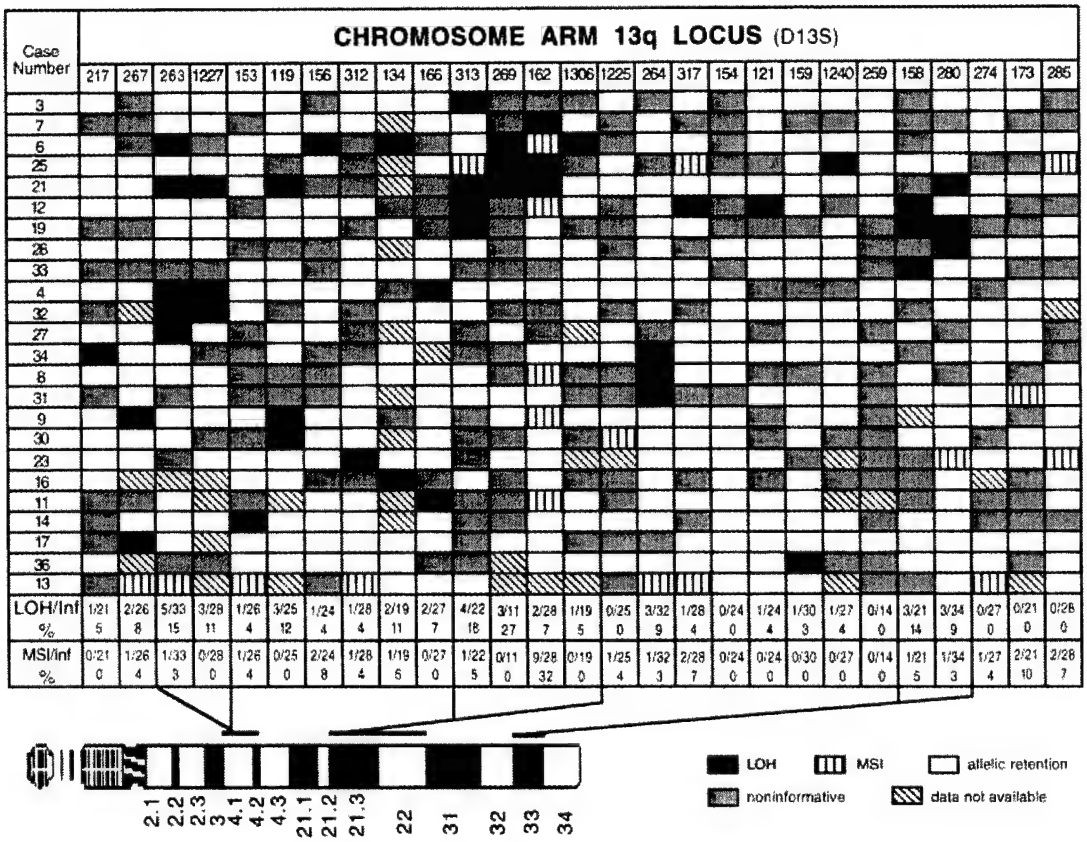

Figure 2. Results of microsatellite analysis for chromosome 13 in primary prostate tumors. Marker numbers are listed at the top, and case numbers are on the left. Only tumors showing LOH or MSI are illustrated. Frequencies of total loss of heterozygosity (LOH) and microsatellite instability (MSI) in informative cases (Inf) for each of the 27 microsatellite markers for all 40 primary tumors are listed at the bottom. The three distinct LOH regions are indicated by bars above the ideogram of chromosome 13.

and one with MSI) at the informative loci. Two of them (cases 42 and 46) had apparently lost the entire chromosomal arm, because all informative loci showed LOH. In addition, two of the 11 tumors (cases 44 and 49) showed MSI for at least 40% of the markers, presenting a replication error phenotype.

DISCUSSION

CGH studies have demonstrated that loss of 13q occurs commonly in prostate cancer. In order to define the distribution and extent of 13q loss, we performed deletion mapping for prostate cancer by using 27 microsatellite markers encompassing the entire q arm. In primary tumors, allelic loss for at least one marker was found in 23 (58%) cases. Half of all LOH was detected at six markers (*D13S263*, *D13S313-D13S269-D13S162*, and *D13S158-D13S280*), which are derived from three regions at q14, q21-22, and q33, respectively.

We found that the primary tumors that had LOH in at least one of the three regions were diagnosed in younger patients and slightly more frequently were of a higher pathologic stage at prostatectomy than were those tumors lacking LOH at these loci. Thus, genetic abnormalities in these chromosomal regions may define a subset of clinically significant primary tumors. Younger age at diagnosis and higher stage are also characteristics of familial prostate cancer (Gronberg et al., 1997). It is unknown, however, whether the 13q loci identified in this study are also important in the development of hereditary prostate cancer.

Frequently deleted loci at 13q covering the potential TSG regions in this study have been found in B-cell chronic lymphocytic leukemia (Kala-

TABLE 1. Clinicopathologic Features of 40 Primary Prostate Tumors and Frequencies of Loss of Heterozygosity (LOH) and Microsatellite Instability (MSI) in Informative (Inf) Cases

Case no.	LOH/ Inf	%	MSI/ Inf	%	Gleason score	Age	Stage
1	0/18	0	3/18	17	7	60	pT3a
2	0/18	0	2/18	11	5	74	pT2b
3	1/18	6	0/18	0	6	47	pT3b
4	3/22	14	0/22	0	7	49	pT2b
5	0/17	0	0/17	0	6	72	pT2b
6	5/20	25	1/20	5	6	60	pT3a
7	0/13	0	1/13	8	7	71	pT3b
8	1/16	6	1/16	7	6	42	pT2b
9	2/21	10	1/21	5	6	60	pT3b
10	0/14	0	1/14	7	7	64	pT2
11	1/13	8	1/13	8	5	61	pT2a
12	4/18	22	1/18	6	6	56	pT2b
13	0/15	0	7/15	47	8	68	pT3b
14	1/18	6	0/18	0	8	69	pT2b
15	0/12	0	0/12	0	7	62	pT2
16	1/12	8	0/12	0	8	60	pT2b
17	1/21	5	0/21	0	7	59	pT3b
18	0/17	0	0/17	0	7	65	pT2b
19	3/14	21	0/14	0	5	50	pT2b
20	0/18	0	0/18	0	6	57	pT2b
21	7/22	32	0/22	0	8	59	pT2a
22	0/13	0	0/13	0	7	62	pT2b
23	1/17	6	2/17	12	6	67	pT2b
25	3/19	16	3/19	16	7	69	pT3b
26	0/14	0	1/14	8	7	61	pT2b
27	1/16	6	0/16	0	9	53	pT3b
28	1/18	6	0/18	0	9	65	pT3
29	0/19	0	0/19	0	6	59	pT2b
30	1/17	6	1/17	6	6	57	pT3a
31	1/16	6	1/16	7	5	67	pT2b
32	2/17	12	0/17	0	7	60	pT2b
33	1/15	7	0/15	0	7	56	pT2b
34	2/18	11	0/18	0	6	65	pT2b
35	0/18	0	0/18	0	7	70	pT3a
36	1/19	5	0/19	0	6	57	pT2a
37	0/14	0	0/14	0	6	55	pT2b
38	0/17	0	1/17	6	6	65	pT2b
39	0/14	0	0/14	0	7	70	pT3a
40	0/14	0	0/14	0	8	70	pT3a
41	0/17	0	0/17	0	7	56	pT3

chikov et al., 1997) as well as in cancers of the lung (Tamura et al., 1997), head and neck (Maestro et al., 1996), liver (Kuroki et al., 1995), kidney (Schoenberg et al., 1995), and ovary (Yang-Feng et al., 1993). In some cancers, deletion of these loci has been associated with a poor prognosis (van den Berg et al., 1996), aggressive behavior (Dotzenrath et al., 1996), and tumor recurrence (Yamaguchi et al., 1996). It is possible that the loci identified in this study are also involved in the development and progression of other cancers.

Our results indicate that allelic loss at the 13q14 region is centered at *D13S263-D13S1227* rather than at the *RB1* locus. The *D13S263* locus, which is 7 cM to the *RB1*, is located in a region that has shown frequent LOH in previous studies of prostate cancer (Cooney et al., 1996; Ittmann and Wieczorek, 1996; Latil et al., 1996). Therefore, although the *RB1* gene seems to play a role in some prostate cancers, another TSG may be located at 13q14.

Based on a recently constructed high-resolution YAC-cosmid-STS map, the *ERCC5/XPG* gene is in the telomeric LOH region, because markers *D13S280* and *D13S158* and this gene were located to the same DNA fragment of 1 megabase (Cayanis et al., 1998). *ERCC5/XPG* is a DNA excision repair gene whose mutations have been detected in patients with xeroderma pigmentosum group G (Nouspikel and Clarkson, 1994). To determine its possible role in prostate cancer, we are currently performing mutation analysis for tumors that showed loss of the telomeric region.

In CGH analyses, 13q loss was observed in 32% of primary tumors, 75% of metastatic lesions, and each of four prostate cancer cell lines. Our results for metastases are in agreement with these CGH findings, as 10/11 (91%) of the cases showed LOH with at least one marker, compared to 23/40 (58%) for primary tumors. Allelic loss at 13q seems to be a frequent event in metastases. It is unknown, however, whether 13q loss is of biological significance and serves as a selection force driving tumor cells toward aggressive behavior, or is simply a result of genetic instability. In the study of Li et al. (1998), the rate of allelic loss at 13q12-q14 was slightly lower in metastases compared to localized tumors. One possible explanation for this inconsistency is that the loci analyzed were different among these studies.

Regions of the *BRCA2*, *RB1*, and *EDNRB* genes, which showed LOH in 8% or fewer of the primary tumors, showed LOH in 56–71% of the metastases. Hence, inactivation of these genes may be more important in metastatic lesions than in primary tumors of prostate cancer. This conclusion is supported by previous studies, in which the *RB1* gene had frequent LOH or loss of expression in aggressive prostate cancers (Melamed et al., 1997; Theodorescu et al., 1997). The *BRCA2* gene had frequent LOH in advanced prostate cancers, and some men with germline *BRCA2* mutations developed and died of prostate cancer (Sigurdsson et al., 1997). Also, a mutation of the *EDNRB* gene occurred in the LNCaP metastatic prostate cancer cell line (Nelson et al., 1998). Three markers at 13q14.1-14.2 and 13q21.1-22 that showed the highest rate of

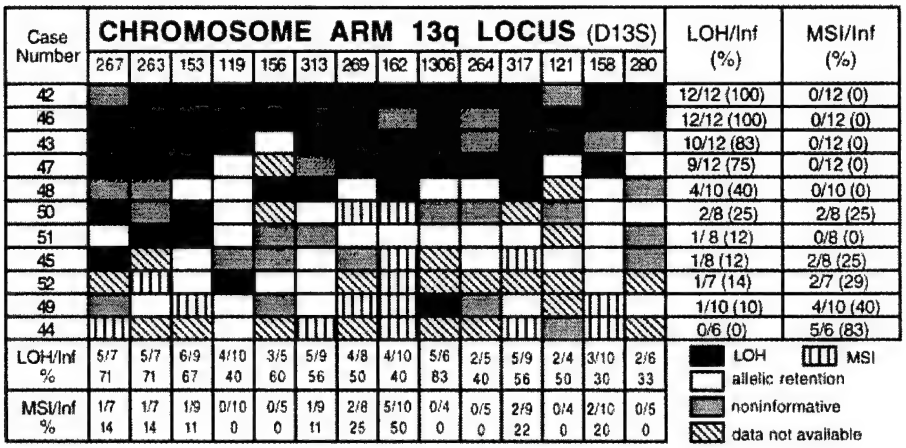

Figure 3. Results of microsatellite analysis for chromosome 13 in metastatic prostate cancer. Marker numbers are listed at the top, and case numbers are on the left. Frequencies of loss of heterozygosity (LOH) and microsatellite instability (MSI) in informative cases (Inf) are listed at the bottom for each of the 14 microsatellite markers and on the right for each of the metastases.

LOH in primary tumors showed an even higher (56–71%) frequency of allelic loss in the metastatic lesions, suggesting that these regions of chromosome 13 harbor genes that are important to both primary and highly aggressive, metastatic prostate cancers.

Along with 13q, chromosomes arms 8p and 16q have shown more frequent LOH in metastases than in primary tumors of prostate cancer. In previous studies, 83% and 61 to 73% of metastatic lesions showed LOH for 8p and 16q (Trapman et al., 1994; Suzuki et al., 1996; Pan et al., 1998), respectively, which are comparable to the LOH rate (91%) for chromosome arm 13q in our study. These data suggest that these chromosomes contain genes associated with advanced prostate cancer.

The frequency of microsatellite instability in prostate cancer has been variable in previous studies (Terrell et al., 1995; Uchida et al., 1995; Watanabe et al., 1995; Cunningham et al., 1996). Our results are consistent with two of the studies in which MSI was detected in about half of the primary tumors studied. However, replication error phenotype due to defective mismatch repair is considered to be present only if at least one third of the examined loci exhibit MSI (Honchel et al., 1995). Based upon this definition, one of the 40 primary tumors and two of the 11 metastatic cancers showed a replication error phenotype. Thus, altered DNA mismatch repair may function in advanced prostate cancer.

In summary, our findings show that, in prostate cancer, the most frequent losses on chromosome 13 are located at 13q14, 13q21-22, and 13q33. Allelic loss at these regions was associated with younger age at diagnosis and might define a subgroup of clinically significant prostate cancer.

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The Prostate 41:190–195 (1999)

Loss of Heterozygosity and Lack of Mutations of the *XPG/ERCC5* DNA Repair Gene at 13q33 in Prostate Cancer

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BACKGROUND. Three regions of chromosome 13 were previously identified for having loss of heterozygosity (LOH) in human prostate cancer. One of them, at 13q33, was defined by LOH at markers D13S158 and D13S280. The *XPG/ERCC5* gene, a DNA repair gene that when mutated in the germline leads to xeroderma pigmentosum, has been mapped to 13q33, within one megabase of D13S158 and D13S280. This paper describes LOH and mutational analysis of the *XPG* gene in human prostate cancers, in order to determine whether the *XPG* gene is involved in the development of prostate cancer.

METHODS. LOH of the *XPG* gene was analyzed in 40 primary prostate cancers and 14 metastases by using the microsatellite assay, and its mutations were examined in 5 cell lines, 14 metastases, and 8 tumors with LOH at 13q33 by using the single-strand conformation polymorphism (SSCP)-direct DNA sequencing analysis.

RESULTS. Four of the 29 (14%) informative primary tumors and 4 of 8 (50%) metastases showed LOH for the *XPG* gene. Analysis of the 8 tumors with LOH at the 13q33 region, 14 metastases, and 5 cell lines of prostate cancer revealed two polymorphisms but no mutation of the gene. The polymorphism in exon 2 did not change the amino-acid sequence of the XPG protein, but the exon 15 polymorphism altered codon 1104 from histidine to aspartic acid. The two polymorphisms also occurred in individuals without prostate cancer.

CONCLUSIONS. LOH at *XPG* in prostate cancer supports the conclusion that the 13q33 region contains a gene important in the development of prostate cancer, while lack of mutations of the gene suggests that *XPG* is not the target gene involved. *Prostate 41:190–195, 1999.*

KEY WORDS: **XPG; ERCC5; loss of heterozygosity; gene mutations; prostate cancer**

INTRODUCTION

Complex DNA repair mechanisms are responsible for mending various types of DNA damage that are believed to be responsible for the development of 80–90% of human cancers [1,2]. Excision repair is one of the major pathways of DNA repair, occurring in all free-living organisms [3]. Multiple steps are involved

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in excision repair, that include recognition and removal of damaged bases or nucleotides, gap filling, and ligation [3]. Each of the steps requires different enzymes to accomplish, and defects in the enzymes in humans may result in the diseases of xeroderma pigmentosum (XP), Cockayne's syndrome, and trichothiodystrophy [4]. Patients with XP have an extreme sensitivity to solar ultraviolet (UV) light and are at high risk of developing skin cancer. XP is categorized into seven different groups (A–G), each representing a distinct defective gene in excision repair [4,5].

The XP group G (*XPG*, also called *ERCC5* for excision repair cross-complementing group 5) gene encodes a DNA endonuclease which functions to remove the damaged DNA region during the process of excision repair [6–9]. Mutations of *XPG* have been detected in patients with XP group G and in those with Cockayne's syndrome [10–12]. Cells from patients with XPG are deficient in repairing UV-induced DNA damage, and expression of the wild-type *XPG* gene in these cells restores their DNA repair capacity [7,13,14]. The *XPG* gene has been cytogenetically mapped to the q33 region of chromosome 13 by fluorescence in situ hybridization [15]. Physically, *XPG* is located near markers D13S158 and D13S280, because 1-megabase DNA fragment contains both these markers and the *XPG* gene [16,17]. We demonstrated previously that the 13q33 region involving markers D13S158 and D13S280 has frequent loss of heterozygosity (LOH) in human prostate cancer, suggesting that a gene in the 13q33 region is inactivated during the development of prostate cancer [18]. It is unknown, however, whether *XPG* shows LOH and/or is mutated in prostate cancer.

To determine whether *XPG* is the target gene at 13q33 involved in prostate cancer, we analyzed LOH and mutations of the *XPG* gene in both primary tumors and metastases of human prostate cancer by using polymerase chain reaction (PCR)-microsatellite analysis and the PCR-single-strand conformation polymorphism (SSCP) method. We detected LOH but no mutation of the *XPG* gene in the cases analyzed.

MATERIALS AND METHODS

Tumors and Cell Lines

Forty pairs of zinc formalin-fixed, paraffin-embedded nonneoplastic tissues and prostate cancer specimens from radical prostatectomy specimens from previously untreated patients were used in this study. Patient age ranged from 42–74 years (median, 60). The pathologic characteristics of the tumors were as follows: Gleason score, 82% had scores of 5–7, while 18% had score 8 or 9; lymph node metastasis, 1 of 40 cases; seminal vesicle invasion, 83% negative and 17% positive; and capsule penetration, 64% negative and 36% positive. The tumors were pathologically staged according to the most recent American Joint Committee on Cancer recommendation [19]. Two thirds were pT2 and one third were pT3 cancers. In addition, 14 metastatic cancer specimens from lymph nodes, liver, or bone and matched nonneoplastic tissues from patients who had died of prostate cancer were obtained at autopsy. Tumor cells were collected from 7 μm (hematoxylin and eosin (H and E)-stained sections, using a previously described protocol for preparation of histological sections on glass slides prior to dissection [20]. Using a microscopic dissection method, tumor samples were selected to ensure a minimum of 70% neoplastic cells. Normal cells from lymph nodes or seminal vesicles (or spleen for autopsy specimens) in almost 90% of the cases, and from nonneoplastic prostate in the remainder, were obtained from paraffin blocks that contained no cancer cells. Five human prostate cancer cell lines were also analyzed for *XPG* alterations. Cell lines LNCaP, PC-3, DU-145, and TSU-Pr1 were purchased from the ATCC (Manassas, VA) and were propagated following ATCC instructions. DNA from the ARCaP cell line was provided by Dr. Haiyen Zhau of the University of Virginia Health Sciences Center [21].

LOH Analysis

DNA was isolated from specimens and cells by adding proteinase K solution, incubating at 55°C for 2–3 days, extracting with phenol and chloroform, and precipitating with ethanol. The *XPG* microsatellite marker, which was identified from intron 8 of the human *XPG* gene [15], was synthesized by Life Technologies (Gaithersburg, MD) and used to analyze allelic status of the gene. Each PCR analysis was performed on 10 ng of genomic DNA, using 1 × PCR buffer (1.5 mM $MgCl_2$); 0.4 μM of each primer; 2 μM of each dNTP; 2.5 μCi of α-^{33}P-dATP (3,000 Ci/mmol) (ICN, Irvine, CA); and 0.5 units of Taq DNA polymerase in a volume of 10 μl. Thirty-five cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 1 min, were performed. PCR products were separated in a 6% denaturing polyacrylamide gel and autoradiographed for 1–3 days. LOH was determined when the signal for one allele in the tumor was reduced significantly when compared to that for the nonneoplastic cells.

PCR Primers for Mutation Analysis

DNA sequences of exons 1 and 2 and their adjacent intron sequences of the XPG gene were available [22], and PCR primers were designed from the intron sequences to amplify these two exons (Table I). Exon/

TABLE I. Primer Sequences From 5′ to 3′ (5′ Primer/3′ Primer), Annealing Temperatures, and PCR Product Sizes Used for PCR Amplification of the *XPG/ERCC5* Gene From Human Genomic DNA*

Exon no.	5′ primer/3′ primer	Temp (°C)	bp
1	GGTGCAGTCCGTCGTAGAAG/AAGTCCCAACGCGGCGTTAA	55	182
2	AACAAGAGTTCAACTAAAAG/AAACTATACGACACTTACCA	50	382
6	GAAGAGTTCTTTCATAATCCTCAAG/CCTCTGGCATTGCTTCAAATAATG	50	144
7	GAGTCTGATGACTTTTCACAGTACC/ATCAAGATGTAATGTGAAGTGTCT	50	203
8.1	GTATTCAAGCTAAGACAGTTGCAG/CAGCCGATCTTTTCTGTCCT	55	623
8.2	TGCAGAGGAGCACGTAGCCA/CATCAGATTCACTTTCTTCCGAG	55	596
9	GAAGTTTCATTGAAGTGCAAAGTGTG/ATATCTTGCCATTCATGGAGCG	55	238
11	GAACTCCTGCGCCTGTTCGG/CCAATTGATTGTGAAAGTCCACAT	55	214
13	AGAATGGTGGCATGAAGCTCAA/CTCTAATTTTGTCGAGATCAGGT	50	200
15.1	ACACAGCTCCGAATTGATTCCTTC/TCAGCATGTTCACTTGAAGA	50	414
15.2	GGGGAGAGACCTGCCTGCCTCTCAGA/TTACGTCTTTGCGACAAATTCATT	55	382

*Temp, temperature; bp, PCR product sizes in base pairs.

intron boundaries for the remaining exons of *XPG* were inferred by comparing human *XPG* cDNA (GenBank accession no. X69978) to mouse *XPG* cDNA (GenBank accession no. D16306) and the mouse genomic sequences [23]. The cDNA sequences of human and mouse *XPG* were compared and aligned using the GAP function of the Wisconsin Package version 9.1, Genetics Computer Group (GCG) (Madison, WI). The nucleotides at each of the exon junctions of mouse *XPG* were identical between human and mouse *XPG* genes; therefore, the same junctions were applied to human *XPG* to divide the cDNA into 16 exon fragments. PCR primers were designed from sequences at both ends of each *XPG* exon, and were used to amplify human *XPG* exons from genomic DNA. Two pairs of primers were designed for exons 8 and 15 due to their large sizes. Exons 3–5, 10, 12, and 14, which constitute 13% of the human *XPG* cDNA, were very small and were not analyzed for *XPG* mutations. Primer sequences, annealing temperature, and PCR product size for *XPG* exons are listed in Table I. All primers were synthesized by Life Technologies.

PCR-Single-Strand Conformation Polymorphism (SSCP) Analysis

PCRs for the SSCP analysis were performed in two rounds. The first-round reaction contained 5–10 ng of genomic DNA, 1 × PCR buffer, 0.4 μM of each primer, 200 μM of dATP, dCTP, dGTP, and dTTP, and 0.5 units of Taq DNA polymerase; incubation was for 35 cycles of 94°C for 30 sec, 50–55°C for 30 sec, and 72°C for 1 min. The PCR products were diluted threefold, and 1 μl of the dilution was used as the template for the second-round PCR, which was performed under the same conditions as in the first round PCR except that 2 μM of each dNTP, 2.5 μCi of α-^{33}P-dATP (3,000 Ci/mmol), and 20 cycles were used. The ^{33}P-labeled PCR products were electrophoresed at 5 W overnight at room temperature in a 6% nondenaturing polyacrylamide gel in 1 × tris-PIPES-EDTA (TPE) buffer (pH 6.8), as described previously [24]. PCR products for exons 2 and 15 were also analyzed in a 0.25 × MDE gel (FMC BioProducts, Rockland, ME) containing 10% glycerol, which were also run at 5 W overnight at room temperature. After electrophoresis, the gels were dried and exposed to Kodak (Rochester, NY) BioMax MR film for 1–2 days. Wild-type and mutant exon 2 of the *PTEN* gene [25] was amplified from normal human DNA and LNCaP DNA, respectively, and the PCR products were run as positive controls along with samples to indicate that sequence variation at a single base in a DNA fragment could be detected in the SSCP gels. The PCR-SSCP procedures were repeated from genomic DNA for samples which showed a band shift, in order to exclude the possibility of PCR artifact.

DNA Sequencing

For the samples which had a band shift in the SSCP analysis, their first-round PCR products were amplified in a volume of 100 μl under the same conditions as the first-round PCR. These PCR products were purified by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI), and were sequenced by using the TaqTrack Sequencing System (Promega), following the manufacturer's instructions. Sequencing data were collected and analyzed by using ScanDNASIS and MacDNASIS software (Hitachi Software, San Bruno, CA).

RESULTS

LOH at the *XPG* Locus

The *XPG* microsatellite marker was analyzed in 40 primary tumors and 14 metastases of prostate cancer

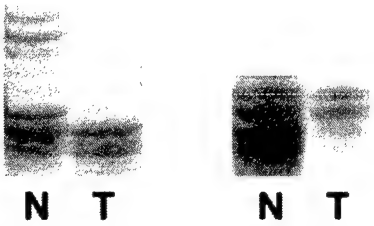

Fig. 1. Loss of heterozygosity of the *XPG/ERCC5* gene in two cases of human prostate cancer, as detected by a microsatellite marker from intron 8 of the gene. N, matched nonneoplastic DNA; T, tumor DNA.

for LOH. Four of the 29 (14%) informative primary tumors showed LOH at the *XPG* locus. In the metastases, 4 of 8 (50%) informative cases had LOH. Examples of LOH in two cases are shown in Figure 1.

SSCP Analysis

In order to determine whether the *XPG* gene was mutated in prostate cancer, we analyzed the 4 primary tumors which showed LOH at the *XPG* locus, 4 additional primary tumors which showed LOH at *XPG*-linked loci D13S158 and D13S280 [18], 14 metastases of prostate cancer, and 5 cell lines derived from prostate cancer metastases for mutations in nine exons of *XPG* by using PCR-SSCP and direct DNA sequencing. The nine exons analyzed constituted approximately 90% of the coding region of the *XPG* gene. SSCP analysis demonstrated that exons 2 and 15 of *XPG* had variant SSCP bands, suggesting that these two exons had sequence alterations. Matched nonneoplastic cells for each tumor were then analyzed using the same procedure, and all but one (case 50) sample showed the same sizes as did the corresponding tumors. In case 50, the nonneoplastic tissue showed two patterns of bands for exon 15, while the tumor showed only one of the patterns (Fig. 2). These results indicate that different sizes of SSCP bands for exons 2 and 15 represent polymorphisms of the gene, and that case 50 had an LOH of *XPG* in the tumor. In the 14 tumors whose DNA was successfully amplified for exon 2, 6 cases (43%) showed heterozygosity, 6 cases (43%) showed homozygosity for the smaller-band allele, and 2 (14%) showed homozygosity for the larger-band allele (Fig. 2). Similarly for exon 15, 5 of 15 cases (33%) showed heterozygosity, 9 (60%) showed homozygosity for the smaller-band allele, and 1 (7%) showed homozygosity for the larger-band allele (Fig. 2). To verify that the polymorphisms in exons 2 and 15 also occur in normal individuals and to determine the frequencies of these polymorphisms, we analyzed 27 DNA samples from unrelated healthy individuals for the two exons. For

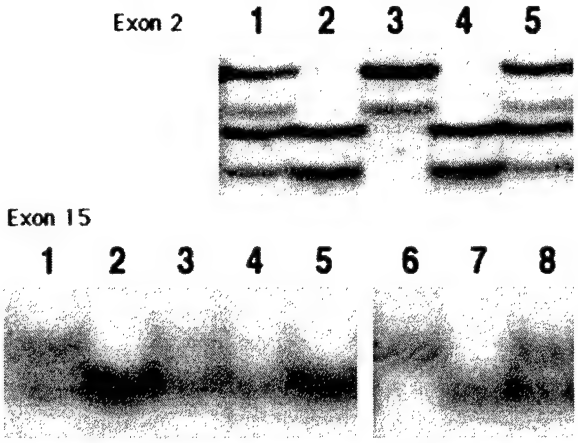

Fig. 2. Different band patterns of polymorphisms for exons 2 and 15 of the *XPG/ERCC5* gene detected by PCR-SSCP analysis. **Lanes 1–5,** cell lines LNCaP, PC-3, ARCaP, DU-145, and TSU-Pr1, respectively; **lanes 6–8,** three tumor cases.

exon 2, 7 individuals (26%) demonstrated a heterozygous phenotype, 9 (33%) were homozygous for the smaller-band allele, and 11 (41%) were homozygous for the larger-band allele. For exon 15, the corresponding allelic frequencies in normal healthy individuals were 37% (10 individuals), 59% (16 individuals), and 4% (1 individual).

Sequencing of Variant SSCP Bands

We determined the DNA sequence of the variant bands detected for exons 2 and 15 in tissues and cell lines. Exon 2 was amplified from the five cell lines, since each of the cell lines contained either the upper allele or the bottom allele or both. Direct sequencing of the PCR products revealed a sequence variation at nucleotide 335 (codon 46) of the *XPG* gene (GenBank accession no. X69978), with a thymine (T) in the allele showing larger bands in SSCP analysis and a cytosine (C) in the allele showing smaller band. ARCaP showed a T at this position, as in the reported sequence (GenBank accession no. X69978), while PC-3 and DU-145 had a C, and LNCaP and TSU-Pr1 showed both C and T (Fig. 3). Similarly, exon 15 was also reamplified and sequenced, and a C→G sequence change at nucleotide 3508 of the *XPG* gene was identified (Fig. 3). This alteration occurred in codon 1104 and resulted in a His→Asp amino-acid substitution. The allele showing larger bands in SSCP analysis contained a C, and the allele showing smaller bands contained a G. Since DNAs from healthy individuals showed the same band shifts in SSCP analysis, we also amplified and sequenced some of the normal DNA. Homozygosity for C or G and heterozygosity of C/G

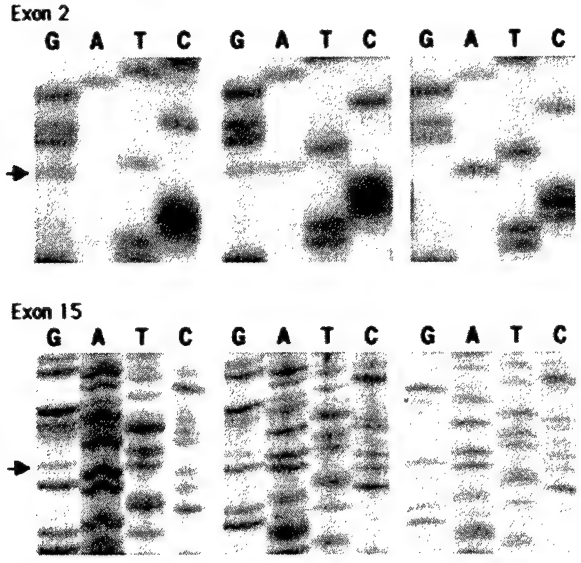

Fig. 3. Sequence analysis of exons 2 and 15 of the *XPG/ERCC5* gene, as primed by reverse primers. Arrows indicate the nucleotide position where polymorphisms occurred. **Left:** Homozygosity for one allele. **Center:** Heterozygosity for two alleles. **Right:** Homozygosity for the other allele.

were found in all the analyzed samples. In case 50, nonneoplastic cells showed heterozygous C/G alleles, but the corresponding tumor cells showed only the G allele, indicating that LOH had occurred.

DISCUSSION

Deletion of chromosome 13 is one of the most frequent genetic alterations in prostate cancer. In this study, we analyzed 62 prostate cancer specimens and cell lines for LOH and mutational status of the *XPG* DNA repair gene, which is located in a previously identified region of LOH at chromosome 13q33 [18]. Using a microsatellite marker isolated from the eighth intron of the gene [15], LOH was found in 14% of the informative primary prostate cancers and 50% of the informative metastases. These LOH frequencies are comparable to those found for the polymorphic markers defining the q33 region (i.e., D13S158 and D13S280) in our previous study [18], that had LOH in 9–14% of the primary tumors and 30% of metastatic tumors. Combining the current study with the previous one [18], 8 tumors had LOH at one or more of the 3 loci in 39 primary tumors which were informative for at least one of the loci, i.e., 8 of 39 (21%) primary tumors had LOH at the 13q33 LOH region. Similarly for the metastases, 7 of 13 (54%) had LOH at this region. Previous studies from other investigators also demonstrated loss at chromosome 13 in prostate cancer and in other malignancies. For example, comparative genomic hybridization revealed loss of 13q in prostate cancer [26–28], and LOH of 13q33 has occurred in ovarian cancer and in head and neck carcinoma [29,30]. These results support the conclusion that there may be a gene located in 13q33 which is important in the development of prostate cancer and some other cancers. However, the target gene in this region has not been identified.

Among the tumors with LOH for any of the three markers (XPG, D13S158, and D13S280), one case had LOH for both XPG and D13S280, and one case had LOH for both D13S280 and D13S158 but not for XPG. Considering that D13S280 is telomeric to D13S158 and both markers are present in the same 1-mb DNA which contains the *XPG* gene [16,17], this finding suggests that the order of the three markers from centromere to telomere is D13S158→D13S280→XPG, and that the 13q33 LOH region likely resides between D13S280 and XPG. Therefore, it is possible that the *XPG* gene is not the target gene at the 13q33 region.

To further determine whether *XPG* plays a role in the development of prostate cancer, we analyzed almost 90% of its coding region in 8 primary tumors with LOH for at least one of the three markers, 14 metastases, and 5 prostate cancer cell lines, using SSCP and direct sequencing methods. No somatic mutation of the gene was detected in any of the samples. This result indicates that mutation of *XPG* is an infrequent event in human prostate cancer, despite the fact that *XPG* is a DNA repair gene and its mutation causes skin cancer in xeroderma pigmentosum [4]. As D13S280 appears to be closer to the center of the 13q33 LOH region relative to XPG and D13S158, this finding also supports the idea that the target gene in the 13q33 LOH region may be located between D13S280 and XPG rather than at the XPG locus.

In our analysis for *XPG* mutations, two polymorphisms, one in exon 2 and the other in exon 15, were identified. The exon 2 polymorphism did not change the amino-acid sequence of the XPG protein, but the exon 15 polymorphism, which occurred at nucleotide position 3508 of the *XPG* gene [13], changed codon 1104 from histidine in one allele to aspartic acid in the other. It is unknown, however, whether this polymorphism affects the repair capacity and/or fidelity of the *XPG* gene and whether it may contribute to the development of prostate cancer. Others have suggested that variation in DNA repair is a factor in cancer susceptibility [31,32]. It might be interesting to analyze the frequencies of the exon 15 polymorphism in larger populations of individuals with and without prostate cancer. We noticed that these polymorphisms were also detected in another study [12].

In conclusion, LOH of the *XPG* gene occurs in hu-

man prostate cancer, but no mutations of this gene could be detected in this study, suggesting that the *XPG* gene is not involved in human prostate cancer and that the target gene at the 13q33 LOH region remains to be identified.

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Deletion at 13q21 Is Associated with Aggressive Prostate Cancers[1]

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ABSTRACT

Previous cytogenetic and molecular genetic analyses suggest that the q21 band of chromosome 13 harbors a tumor suppressor gene(s) involved in prostatic carcinogenesis. The precise genetic location, however, has not been defined. In this study, we examined prostate cancer specimens and cell lines/xenograft for genetic deletions at 13q21, using the methods of tissue microdissection and duplex PCR. Deletions at 13q21 were detected in 13 of 147 (9%) prostate cancer samples. Deletion of the same region was also detected in the LNCaP cell line and the PC-82 xenograft of prostate cancer. The overlapping region of deletion in LNCaP and PC-82 spans 3.1 cM or 2.9 cR, which is equivalent to 1–3 Mb. The endothelin receptor B gene, a possible tumor suppressor gene at 13q21, was not located in the region of deletion. Among the 13 prostate neoplasms with deletion at 13q21, 5 were metastases, and 7 were poorly differentiated primary tumors. The only primary tumor that was not poorly differentiated but had deletion occurred in one of the youngest patients (49 years) at diagnosis. These results provide evidence that 13q21 may harbor an unidentified gene(s) whose inactivation occurs in some aggressive carcinomas of the prostate. In addition, this study provides a framework for the cloning and identification of the 13q21 gene(s).

INTRODUCTION

Molecular determinants important in the development and progression of prostate cancer are poorly understood, despite the fact that this neoplasm has become a significant health problem (1). Cytogenetic and molecular genetic analyses have indicated that interstitial deletions on chromosomes 8p, 13q, 10q, 6q, 7q, 17q, and 18q occur frequently in human prostate cancer, suggesting the existence of tumor suppressor genes on these chromosomal arms (2, 3). At present, however, only 10q has been identified as having a tumor suppressor gene (*i.e., PTEN*) that has been implicated in prostate cancer (4, 5). The target genes from the remaining chromosomes have yet to be mapped and identified.

Deletion of portions of chromosome 13 has been detected by various genetic approaches in human prostate cancer. In a cytogenetic banding study, nonrandom loss of chromosome 13 was observed in a xenografted cell line (6). CGH[3] demonstrated that loss of 13q is the second most frequent chromosomal alteration, having occurred in 32% of primary tumors, 56–75% of recurrent and metastatic tumors, and each of the four commonly used prostate cancer cell lines derived from metastatic prostate cancer (7–11). One CGH study suggested a deletion region at the q21 band of chromosome 13 (10). In our LOH assay, we identified a distinct region of LOH in a 7-cM DNA segment involving markers D13S269 and D13S162 at 13q21 (12). These studies suggested that a tumor suppressor gene is located at 13q21; however, the DNA segment containing this gene was still too large for its identification.

To fine map the region of deletion and to evaluate the clinical significance of 13q21 deletion in prostate cancer, we analyzed a number of STS markers at 13q21 for genetic deletions in prostate cancer, using the approaches of tissue microdissection and duplex PCR. The minimal region of the deletion was confined to a DNA fragment of 3.1 cM or 2.9 cR (1–3 Mb), and the deletion at 13q21 appeared to be associated with tumor aggressiveness.

MATERIALS AND METHODS

Tumor Specimens, Cell Lines, and Xenograft. A total of 147 prostate cancer tissues from 125 patients were examined for deletion in this study. Of them, 103 were primary tumors, 6 were lymph node metastases obtained at surgery, and 38 were either primary tumors (12 specimens) or metastases (26 specimens) from various organ sites obtained at autopsy from 16 patients who died of prostate cancer. Among the 103 primary tumors from surgery, one was a well-differentiated tumor (Gleason score, 4), 19 were moderately differentiated cancers (Gleason score, 5 or 6), 40 were moderately poorly differentiated tumors (Gleason score, 7), and 43 were poorly differentiated neoplasms with Gleason scores of 8–10. Each of the primary tumors from autopsy was a high-grade cancer. Patient age ranged from 42–88 years. Tumor tissues were zinc formalin-fixed and paraffin-embedded, and the cells for DNA isolation were collected from 7-μm H&E-stained sections using a previously described protocol for preparation of histological sections on glass slides before microdissection (13), which ensured a minimum of 70% neoplastic cells. Nonneoplastic cells from lymph nodes or seminal vesicles in most of the cases or from normal prostate stroma or urothelium in the remainder of the cases were obtained from paraffin blocks that contained no neoplastic cells.

Prostate cancer cell lines LNCaP, PC-3, DU 145, and TSU-Pr1 were purchased from American Type Culture Collection (Manassas, VA) and propagated following the manufacturer's instructions. The PC-82 prostate cancer xenograft was described previously (14).

DNA Preparation. For most of the tumor specimens, DNA was isolated from microdissected cells by adding proteinase K solution and incubating at 55°C overnight, followed by boiling the solution for 10 min to inactivate proteinase K, as described previously (12). One μl of DNA sample was used in each PCR. For tumor specimens including all of the autopsy cases where greater amount of tissues were available, phenol/chloroform extraction and ethanol precipitation were performed after the proteinase K treatment. For these samples, 5–50 ng of DNA were used for each PCR. For the cell lines and the PC-82 xenograft, genomic DNA was isolated by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

STS Markers. We initially analyzed the two mapped genetic markers, *i.e.*, D13S269 and D13S162, that are located in the 13q21 LOH region in our previous study (12). After deletion for either of these markers was detected, additional markers that flank the deleted ones were analyzed to further define the segment with deletion. Selection of additional markers was based on the latest version of the integrated human genomic map (15),[4] which is available on line from the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research, Human Genetic Mapping Project and from the high-resolution yeast artificial chromosome-cosmid-STS map of human chromosome 13 (16). Primer sequences of these markers are available from the Genome Database.[5] The endothelin receptor B (*EDNRB*) gene, which is close to but telomeric to marker D13S162 at 13q21 (17), was also examined using a STS marker derived from its fourth exon (18). Primer sequences for exon 4 of the *EDNRB* gene are 5′-ATCCCTATAGTTTTACAAGACAGC-3′ (for-

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[3] The abbreviations used are: CGH, comparative genomic hybridization; LOH, loss of heterozygosity; STS, sequence tagged site.

[4] Supplementary data from the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research, Human Genetic Mapping Project (http://www-genome.wi.mit.edu).

[5] http://gdbwww.gdb.org.

ward) and 5′-ATTTTCTTACCTGCTTTAG GTG-3′ (reverse). PCR primers were either purchased from Research Genetics (Huntsville, AL) or synthesized by Life Technologies, Inc. (Gaithersburg, MD).

In addition to 13q21 markers, each PCR contained one internal control STS marker, *i.e.*, one of the exons from the KAI1 gene whose deletion has not been found in prostate cancer (19, 20). Internal controls were necessary for reliable detection of chromosomal deletions. Depending on the size of PCR products of a 13q21 marker, exon 5, 7, or 8 of the KAI1 gene was used. The primer sequences of these KAI1 exons have been described previously (19). Sizes of PCR products, annealing temperatures, KAI1 control exons, genetic and physical maps, and deletion status in LNCaP and PC-82 cells for the 13q21 markers are listed in Table 1.

Deletion Analysis. The duplex PCR approach was used for the deletion detection. Each PCR, which was in a volume of 10 μl, contained 1 μl of genomic DNA, 1× PCR buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA], 0.4 μM of each primer, 20 μM of each deoxynucleotide triphosphate, 1 μCi of [α-^{33}P]dATP (3000 Ci/mmol; ICN, Irvine, CA), 0.6 unit of platinum Taq antibodies (Life Technologies, Inc.), and 0.6 unit of Taq DNA polymerase. After an initial denaturation at 95°C for 5 min, 30 cycles, each consisting of denaturation at 94°C for 45 s, annealing for 45 s, and extension at 72°C for 1 min, were performed.

PCR products were separated in 5% nondenaturing polyacrylamide gels (size, 20 × 20 cm) at 200 V for 1–2 h. The gels were dried and exposed to Kodak Biomax MR film at room temperature overnight. Absence of a PCR product for a 13q21 marker in a tumor defined a deletion. However, the microdissected tumor samples were sometimes contaminated with nonneoplastic cells sufficient to give rise to PCR products. When this occurred, signal intensities for PCR products were quantitatively measured by scanning and analyzing PCR bands from a film using ScanDNASIS software (Hitachi Software, San Bruno, CA); the ratio of signal intensity of a 13q21 marker to that of the internal control marker was calculated for each DNA sample, and a deletion was considered to be present when such a ratio in a tumor was less than half of that in its matched nonneoplastic cells.

All experiments were repeated one to three times, and the deletions were detected in each of the experiments.

RESULTS

Based on the multiplex PCR method used for the detection of homozygous deletion in our previous study (21), we first adjusted the experimental procedures to maximize the sensitivity and consistency in detecting genetic deletion. Compared with regular PCR and agarose gel electrophoresis, we found that the procedure of radioactive PCR with [α-^{33}P]dATP, use of the hot-start approach by adding Taq antibodies, separation of PCR products with nondenaturing PAGE, and exposure of gels to Kodak Biomax MR film was more consistent, quantitative, and sensitive in demonstrating genetic deletions in tumor samples.

We first analyzed the two markers (*i.e.*, D13S269 and D13S162) that were located in the LOH region at 13q21, based on our previous study (12), in each of the 147 tumor specimens using the improved method of duplex PCR assay (Table 1). Some tumors showed absent or reduced band intensities for D13S269 and/or D13S162 compared with that of the internal control marker and with that of a normal control. We then repeated the PCR for these tumors, along with their matched nonneoplastic cells. As shown in Fig. 1 for some specimens, deletions at D13S269 and D13S162, which could be either homozygous deletions or hemizygous deletions, were repeatedly detected in 13 tumor specimens. Whereas 11 of 13 tumors lost both D13S269 and D13S162, 2 tumors lost D13S269 only (Table 2).

In total, 13 of 147 (9%) prostate cancer samples showed deletion at 13q21. Patient age at diagnosis and Gleason score for these neoplasms are shown in Table 2. Among these tumors, five were metastases, and seven were poorly differentiated primary tumors. The only primary tumor that was not poorly differentiated but had a deletion (case 233) occurred in one of the youngest patients (49 years) at diagnosis.

To determine whether deletion at 13q21 also occurred in prostate cancer cell lines and xenograft, we analyzed D13S269 and D13S162 for deletion using the same duplex PCR method. As shown in Table 1 and Fig. 2, whereas three cell lines did not show any deletions, the LNCaP cell line and the PC-82 xenograft showed a significant signal reduction at D13S269 and D13S162. Measurement of signal intensities using the ScanDNASIS program indicated that the signal ratio of the deleted marker to the internal control in LNCaP cells was only about one-fourth of that in the normal placenta control DNA. Deletion analysis of cell lines and xenograft was also repeated using regular PCR and agarose gel electrophoresis, and deletion of D13S269 and D13S162 was demonstrated in each experiment (data not shown).

Table 1 *Genetic and radiation hybrid (RH) maps, sizes of PCR products, annealing temperatures, internal KAI1 control markers, and deletion status in LNCaP and PC-82 cells for the 13q21 markers*

Marker names[a]	Genetic map (cM)	RH map (cR)	PCR product (bp)	Annealing temperature (°C)	KAI1 exons	LNCaP	PC-82
D13S1273	49.4		115	55	7	+	+
D13S1260	50.7		141	57	7	+	+
D13S1317	51.9		223	57	8	+	+
D13S275	52.7		239	55	7	−	+
D13S1310	53.5		143	55	7	−	+
D13S1318	54.0		259	57	7	−	+
WI-5860		191.5	224	57	8	−	+
WI-3660			132	61	7	−	+
D13S1090E[b]	56.6		107	61	7	−	+
D13S152	56.6		138	55	7	−	−
D13S745			189	57	7	−	−
D13S791			294	61	7	−	−
D13S1249	57.3		220	60	8	−	−
D13S166	57.3		115–125	57	7	−	−
D13S156	57.3	193.2	272–286	60	7	−	−
D13S269	58.3		142	60	7	−	−
H65656	59.7	194.4	~150	61	7	−	−
D13S162	59.7	194.4	182–202	55	7	−	−
BS610/611[b]	59.7	194.4	95	57	7	+	−
D13S1562			148	60	7	+	−
WI-16413			132	60	7	+	−
D13S160	62.7	207.7	229–241	57	8	+	+
EDNRB			170	60	5	+	
D13S170	65.4		113	57	7	+	+

[a] The markers are listed in order from centromere to telomere according to the published genomic maps (15, 16).[4]

[b] Markers D13S1090E and D13S152 are located in one BAC clone and are thus considered to be at the same genetic location (data not shown). Similarly, markers BS610/611, H65656, and D13S162 are in another BAC clone. Primers sequences for marker BS610/611 are 5′-TATTCATCCAGCCCCCTCAATG-3′ and 5′-AGATGTGCAGGAGATGAATGGC-3′.

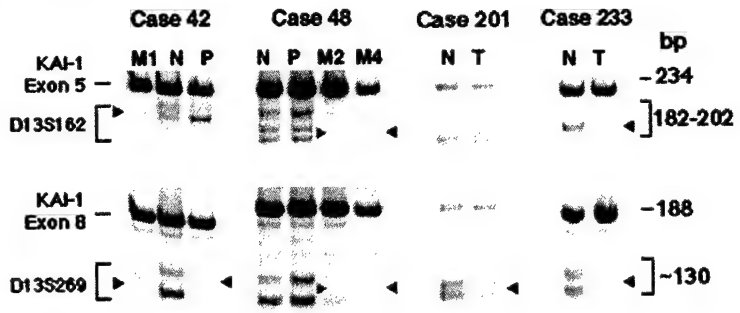

Fig. 1. Detection of deletion at 13q21 in prostate cancer by duplex PCR assay. Case number and tissue type are indicated at the *top*, STS markers are indicated at the *left*, and the size of the PCR products is indicated at the *right*. Each *arrow* denotes a deletion at a marker in a neoplasm. *Lanes P* and *M*, primary tumor and metastasis obtained from autopsy specimens; *Lane T*, primary tumor obtained at surgery; *Lane N*, matched nonneoplastic cells in each case. Due to the polymorphic feature of microsatellite markers, two bands that represent two alleles are seen for both markers in most cases. Case 42 (*Lane P*) appears to have LOH instead of deletion at D13S162.

To determine the size of the region with deletion, we selected more STS markers (Table 1) that flank D13S269 and/or D13S162 at 13q21 and examined them in LNCaP and PC-82 tumor cells that showed deletions at D13S269 and D13S162 and had a sufficient quantity of pure tumor DNA for analysis (Table 1; Fig. 2). The deletion region in LNCaP was different from but overlapped that in PC-82, and the common region of deletion was defined as a DNA segment of 3.1 cM within markers D13S1090E and BS610/611 or 2.9 cR within markers WI-5860 and BS610/611 (Table 1). According to the current genomic maps (15, 16), the size of this common region of deletion was estimated to be 1–3 Mb. The endothelin receptor B (*EDNRB*) gene, which is located at least 3 cM telomeric to the common region of deletion at 13q21 and has been suggested as a tumor suppressor gene, was also analyzed in five tumors and in all of the cell lines but showed no deletion (data not shown).

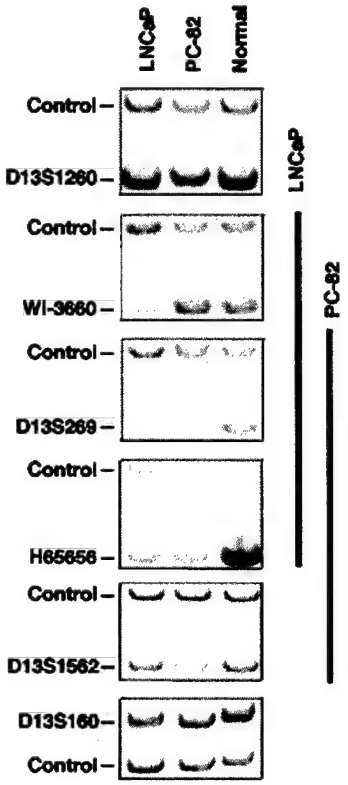

Fig. 2. Deletions of 13q21 markers in LNCaP cell line and PC-82 xenograft of prostate cancer. Sample names are indicated at the *top*, and marker names are indicated at the *left*. Markers deleted are indicated by *vertical bars* at the *right*. Normal DNA was from a normal human placenta.

DISCUSSION

In this study, we first improved the multiplex PCR procedure used in our previous study (21) for the detection of genetic deletion in human tumors. Use of radioactive PCR with a hot-start approach, PAGE, and exposure to Kodak Biomax MR film made deletion detection more reliable when compared with regular PCR and agarose gel electrophoresis. As shown in Fig. 1, some tumor samples had no signal or a very faint signal at the deleted markers D13S269 and D13S162 compared with their matched nonneoplastic cells. Detection of deletion in the LNCaP cell line but not in any other cell lines further indicated the feasibility of our methods because a high-resolution cytogenetic banding study showed that each LNCaP cell has an interstitial deletion at 13q21 in some but not all of its copies of chromosome 13 [it has a near tetraploid karyotype (22)].

Genetic deletion at 13q21 in prostate cancer was first demonstrated by a cytogenetic banding study in the LNCaP cell line (22). In CGH analyses, deletion of 13q was detected in human prostate cancer tissues and in each of the four commonly used prostate cancer cell lines (7–11), and a common region of deletion was suggested to be located at 13q21 (10). Recently, our LOH study further showed that the deletion at 13q21 involved markers D13S313, D13S269, D13S162, and D13S1306 in a DNA interval of 7 cM (12). Using the methods of tissue microdissection and an improved duplex PCR assay, we found that genetic deletions at 13q21 occurred in 13 of 147 (9%) prostate cancer specimens and 2 of 5 (40%) prostate cancer cell lines and xenograft. Consistent with previous studies, our data provide additional evidence for the existence of a tumor suppressor gene(s) at 13q21.

Although previous studies identified a region of deletion at 13q21 in prostate cancer, the size of the region with deletion was still too large for gene identification, and the precise location of the target gene remained to be defined. Using densely mapped genetic markers and the LNCaP cell line and PC-82 xenograft of prostate cancer, we defined the common region of deletion to be in a 3.1-cM segment within markers D13S1090E

Table 2 *Prostate cancers showing deletions at D13S269 and D13S162 and patient age at diagnosis and Gleason score (G.S.). "M" indicates a metastasis*

Presence or absence of a marker in a tumor is indicated by + or −, respectively.

Case no.	Age (yrs)	G.S.	D13S269	D13S162
42-P	68	9	−	+
42-M1	68	M	−	−
48-M2	71	M	−	−
48-M4	71	M	−	−
53-P	67	9	−	−
53-M3	67	M	−	−
104-T	75	9	−	−
110-T	65	9	−	−
122-M	68	M	−	−
200-T	71	8	−	−
201-T	80	10	−	+
228-T	69	9	−	−
233-T	49	7	−	−

and BS610/611 and a 2.9-cR segment between WI-5860 and BS610/611 (Table 1). Considering that, on average, 1 cM is equivalent to 1 Mb and 1 Mb is equivalent to 3.7 cR for the Genebridge4 Radiation Hybrid Panel,[6] our findings indicate that the minimal region of deletion containing the target gene should be in the size range of 1–3 Mb.

Twelve of the 13 prostate cancer samples with homozygous deletion at 13q21 were either poorly differentiated primary tumors or metastases. The only primary tumor that was not poorly differentiated but had a deletion occurred in one of the youngest patients (49 years) at diagnosis (Table 2). The LNCaP cell line and the PC-82 xenograft, which were noted above to have a deletion at the same region of 13q21, were also derived from metastases of prostate cancer (23). These data indicate that deletion at 13q21 occurs in biologically aggressive prostate cancers. Consistently, a previous study found that patients whose prostate cancers showed LOH at 13q were diagnosed at a significantly younger age than those whose tumors lacked LOH at 13q (12).

The endothelin receptor B (*EDNRB*) gene is located at 13q21, telomeric to marker D13S162 but centromeric to marker D13S160 (17). Considering that promoter methylation of the *EDNRB* gene occurs frequently in prostate cancer and that this gene has been suggested to act as a tumor suppressor (24), we analyzed its deletion status in five tumors that showed deletion at D13S269 and D13S162 and in all of the prostate cancer cell lines. No deletion at the *EDNRB* gene was detected. Based on the current genomic map, *EDNRB* is at least 3 cM telomeric to the common region of deletion. Therefore, *EDNRB* is not the target gene for the 13q21 deletion region in prostate cancer. Currently, there is no gene that is located in the region of deletion that has been identified as a tumor suppressor. This study provides a framework for the identification of this gene.

Genetic deletion involving 13q21 has also been detected by CGH in malignant fibrous histiocytomas (25, 26) and other sarcomas (27, 28), and gliomas (29, 30). These studies suggest that different types of tumors may share the same genetic alteration at 13q21 during carcinogenesis or progression.

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Loss of Heterozygosity at 13q14 and 13q21 in High Grade, High Stage Prostate Cancer

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BACKGROUND. Loss of heterozygosity (LOH) at chromosome 13q has been frequently detected in prostate cancer, and three regions (i.e., 13q14, 13q21, and 13q33) may harbor tumor suppressor genes important in this neoplasm. In this study, we examined the frequency of 13q LOH in advanced prostate cancers, in order to determine the clinicopathologic relevance of 13q LOH.

METHODS. LOH was determined by analyzing microsatellite markers in 41 cases of microdissected predominantly high grade prostate cancer tissues and their matched nonneoplastic cells. The results were compared with those generated previously for lower grade, asymptomatic cancers.

RESULTS. The frequencies of LOH at 13q14, 13q21, and 13q33 were 62% (21/34), 57% (20/35), and 34% (11/32), respectively. In comparison to previous results, LOH at 13q14 and 13q21 but not 13q33 was more frequent in prostate cancers that produced local clinical symptoms (bladder outlet obstruction) than those that did not ($P < 0.05$). LOH at 13q14 was also significantly more frequent in high grade and high stage cancers than those that were lower grade and lower stage ($P < 0.05$).

CONCLUSIONS. Although the target genes on 13q have not been identified in carcinomas of the prostate, LOH at 13q14 in particular is associated with clinically significant prostate cancers. Further fine mapping of these loci may lead to identification of tumor suppressor genes that are deleted in aggressive carcinomas of the prostate. *Prostate 49: 166–171, 2001.*

KEY WORDS: **prostate cancer; LOH; chromosome 13; 13q14; 13q21; 13q33**

INTRODUCTION

Prostate cancer is a significant health problem that kills tens of thousands of American men each year [1]. The molecular determinants of prostate cancer, however, are poorly understood. Unlike cancers of many other organs, only a small proportion of prostate cancers (11%) are clinically apparent, and 3% of them lead to patient death [2]. Therefore, it is important to identify and evaluate the molecular alterations that are responsible for the biologic behavior of prostate cancers, with the intention of better identifying aggressive tumors from those that are unlikely to produce adverse patient outcomes.

Deletion of portions of chromosome 13 occurs in a variety of human cancers including carcinoma of the prostate. In a cytogenetic banding study, deletion of chromosome 13 was observed in a xenografted prostate cancer cell line [2]. Comparative genomic hybridization (CGH) demonstrated that loss of 13q was the second most frequent chromosomal alteration in prostate cancer [3–7]. Loss of heterozygosity (LOH) of chromosome 13 has also been demonstrated in prostate tumors [8–15], and three distinct regions at

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13q14, 13q21, and 13q33, respectively, have been identified [14]. Although the target genes have not been identified for the LOH regions at 13q, it has been shown that LOH at 13q is very frequent in metastases of prostate cancer and is associated with younger patient age at diagnosis in localized prostate neoplasms [14]. These studies indicate that chromosome 13 harbors gene(s) whose inactivation is involved in clinically significant prostate cancers.

In this report, we further analyze LOH at the three previously identified LOH regions in 41 predominantly poorly differentiated primary prostate cancers to determine which of the three loci of 13q are lost most commonly in these clinically important neoplasms.

MATERIALS AND METHODS

Tumor Specimens

Forty-one pairs of matched zinc formalin-fixed, paraffin-embedded nonneoplastic and prostate cancer tissues from transurethral resection specimens were analyzed. Patient age ranged from 52 to 88 years (median, 70 years). While three (7%) tumors had a Gleason score of 6 and six (15%) had a Gleason score of 7, 32 (78%) had Gleason scores of 8–10. The clinical tumor stage and follow-up were recorded by the McIntire Tumor Registry at the University of Virginia Health System. All the patients for the 41 cases have died with disease. Tumor cells for DNA isolation were collected from 7 μm H and E-stained sections using a previously described protocol prior to microdissection [16], which typically ensured a minimum of 70% neoplastic cells for each sample. Nonneoplastic cells collected were present on the same slides as cancer cells and included stromal cells, lymphocytes, and urothelium; in most cases, they did not include nonneoplastic prostatic epithelium.

Selection of Markers

The three LOH regions at 13q14, 13q21, and 13q33 identified previously [14] were numbered as regions one, two, and three, respectively. Two markers for each of the three LOH regions, i.e., D13S263 and D13S1227 for region one, D13S269 and D13S162 for region two, and D13S158 and D13S174 for region three, were selected for LOH analyses. In addition, three markers that flank regions one and two but are not located in any of the LOH regions, i.e., D13S1253 at 13q14 and D13S166 and D13S170 at 13q21, were also analyzed to monitor the background level of LOH. Chromosomal location, genetic map in cM, size of PCR products, and annealing temperature for each of the markers analyzed are listed in Table I. Primer sequences of these markers are available from the Genome Database (http://gdbwww.gdb.org). All the markers were purchased from Research Genetics (Huntsville, AL).

LOH Assay

DNA was isolated from specimens by adding proteinase K solution, incubating at 55°C overnight, followed by boiling the solution for 10 min to inactivate proteinase K [14]. One microliter of DNA sample was used in each PCR, that was performed on 1 X PCR buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton® X-100, 0.1 mg/ml BSA); 0.4 μM of each primer; 2 μM of each dNTP; 1 μCi of α-^{32}P-dCTP (3000 Ci/mmol); and 0.6 unit of Taq DNA polymerase in a volume of 10 μl. Thirty or 35 cycles, each consisting of denaturation at 94°C for 30 sec, annealing for 30 sec, and extension at 72°C for 1 min, were performed. PCR products were separated in 6% denaturing polyacrylamide gels and autoradiographed for 2–16 hr at room temperature.

TABLE I. Chromosomal Location, Genetic Map (cM) From the Top of Chromosome 13, Size of PCR Products, and Annealing Temperature for Each of the Markers Analyzed in This Study

Markers	Cytogenetic location	Genetic map (cM)	PCR product size (bp)	Annealing temperature (°C)
D13S1253	13q14	36.6	~140	55
D13S263	13q14	40.4	~149	61
D13S1227	13q14	41.7	~134	61
D13S166	13q21	57.3	~118	60
D13S269	13q21	58.3	~130	60
D13S162	13q21	59.7	182–202	61
D13S170	13q21	65.4	~136	55
D13S174	13q33	86.9	~188	55
D13S158	13q33	86.9	99–113	61

LOH was defined when the signal for one of the two alleles was absent in a tumor but present in its matched nonneoplastic cells, while the other allele was present in both normal and cancer cells. Due to the heterogeneous nature of prostate cancer samples, tumor specimens, even when prepared by microdissection, were often contaminated with nonneoplastic cells that sometimes gave rise to a fainter signal in a tumor for a deleted marker. Most such losses, however, were typically identified without difficulty. In the cases in which the presence of LOH was more difficult to interpret, signal intensities for PCR products were quantitatively measured by scanning and analyzing PCR bands for an allele using the ScanDNASIS software (Hitachi Software, San Bruno, CA); the ratio of signal intensity of one allele to the other was calculated for each DNA sample. LOH was considered to be present when the ratio in a tumor was less than half of that in its matched nonneoplastic cells.

For each of the three LOH regions defined previously [14], LOH was considered to be present if at least one of the two markers showed LOH; a normal status was defined if no LOH was observed but at least one marker was informative; and data were not available (NA) if both markers were uninformative, or occasionally one marker was uninformative and the other failed to yield a PCR product or showed shifted bands (microsatellite instability) [14].

Statistical Analysis

The relationship between LOH frequencies and clinicopathologic features of tumor samples was analyzed statistically by the use of χ^2 analysis-of-contingency tables [17]. In correlating 13q LOH with tumor grade and tumor stage, data in the current study was combined with that of the earlier study [14].

RESULTS

We examined LOH status at three previously identified LOH regions that are located at 13q14, 13q21, and 13q33. These loci, numbered as regions one, two, and three, respectively, were assessed in 41 prostate cancer specimens derived from patients who had symptoms of bladder outlet obstruction and who underwent transurethral resection (TUR). The majority of the cancers (78%) were poorly differentiated, showing Gleason score of 8 or above. LOH status at each of the three LOH regions and control markers, Gleason score, and clinical stage for each case are shown in Figure 1. Examples of LOH are shown in Figure 2.

For the three "control" markers that flank regions one and two but are located outside the three LOH regions [14], the LOH frequency was 27% (7/26) at D13S1253 (13q14), 23% (5/22) at D13S166 (13q21), and 25% (6/24) at D13S170 (13q21), respectively (Fig. 1). In combination, the three control markers had LOH at 18 of 72 (25%) polymorphic loci. The majority of these losses, however, appeared to extend from LOH at regions one or two, and only 6/60 (10%) of the

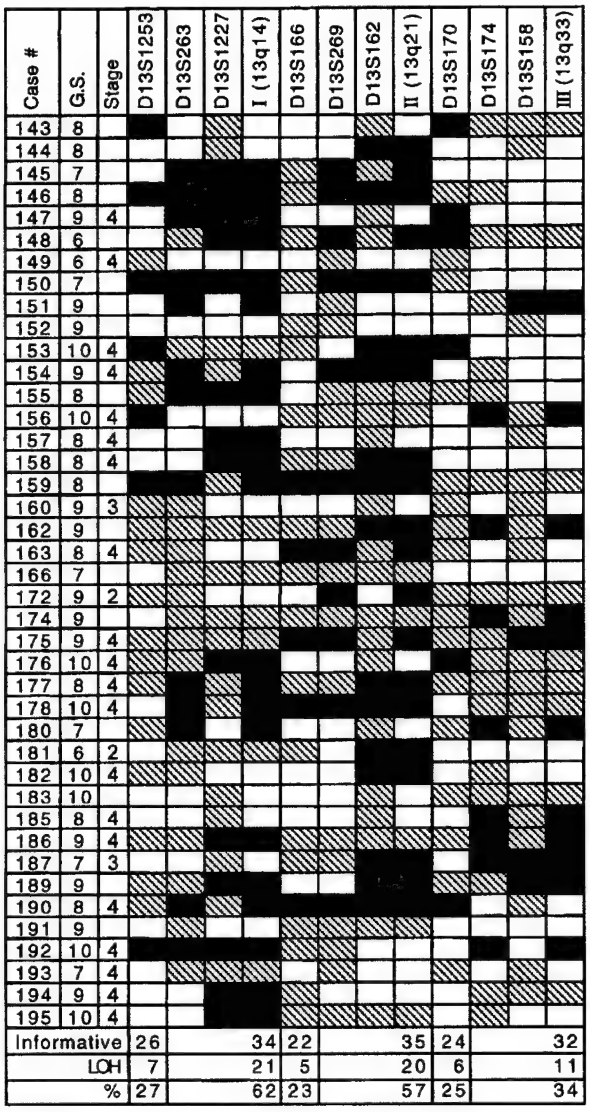

Fig. 1. LOH status for the three LOH regions (i.e., regions I, II, and III at 13q14, 13q21, and 13q33, respectively) and the three control markers (i.e., DI3SI253, Dq13SI66, and DI3SI70) in 41 predominantly high grade prostate cancer tissues. The markers/regions are listed from left to right according to their chromosomal location from centromere to telomere. The number of informative cases and LOH cases and the frequency of LOH are shown at the bottom. G.S., Gleason score; stage is clinical stage at diagnosis; dark square, LOH; hatched square, unknown LOH status; blank area, informative but no LOH.

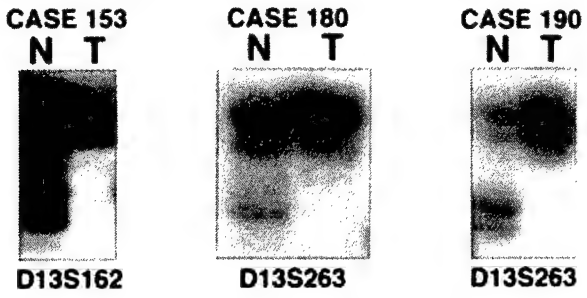

Fig. 2. Examples of LOH in prostate cancers as detected by analyzing microsatellite markers. N and T indicate normal and tumor tissue. Case number is at the top, and marker name is at the bottom of each picture.

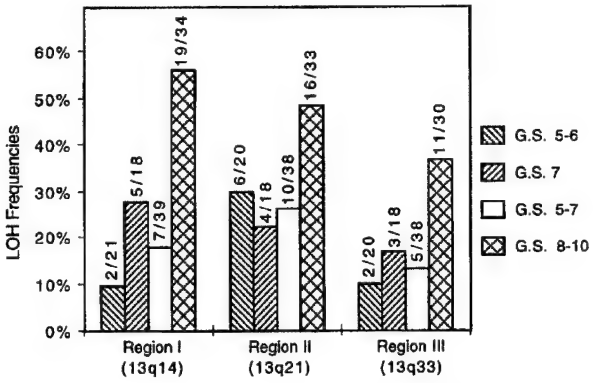

Fig. 3. LOH frequencies at the three LOH regions at 13q14, 13q21, and 13q33, respectively, in prostate cancers of different grades. The number of cases with LOH over the number of informative cases is shown on top of each bar indicating tumor grade.

polymorphic loci showed LOH at the control markers only. The frequencies of LOH at regions one, two, and three were 62% (21/34), 57% (20/35), and 34% (11/32), respectively. Compared to the 10% LOH frequency for control markers, each of the three regions showed significantly more frequent LOH ($P < 0.05$).

In our previous study of cancers present in radical prostatectomy specimens [14], markers D13S263, D13S1227, D13S269, D13S162, and D13S158, which defined the three LOH regions, showed LOH at 15, 11, 27, 7, and 14%, respectively. According to the definition of LOH for each region in this study, LOH occurred in 5/39 (13%), 6/36 (17%), and 5/36 (14%) at regions one, two, and three, respectively. Compared to these results, the tumors in this study had significantly more frequent LOH ($P < 0.05$) at regions one and two but not at region three. One important difference between this study and the previous one was that all the tumors in this study occurred in patients with local clinical symptoms (bladder outlet obstruction), while the tumors used in the previous study were diagnosed by serum PSA test without clinical symptoms. Another difference between the two studies was that the majority of tumors in the current study were high grade (Gleason score of 8 or above) and were stage IV, while the tumors in the previous study had predominantly low to mid grades (Gleason score of 5 to 7) and stages II and III (pathologic stage) [14]. In addition, 32 of 41 (78%) patients from this study died with disease within three years of diagnosis, while at least 36 of 40 patients (90%) from the previous study were still alive at three years after diagnosis [14].

We also combined the results from these two studies and compared LOH frequencies according to tumor grade (Fig. 3). The tumors with high grade (Gleason score of 8 or above) showed LOH frequencies of 19/34 (59%) at region one, 16/33 (48%) at region two, and 11/30 (37%) at region three, while tumors of lower grade (Gleason score of 7 or below) showed 7/39 (18%), 10/38 (26%), and 5/38 (13%) at regions one, two, and three, respectively (Fig. 3). While high grade tumors showed more frequent LOH at each of the three LOH regions, only region one showed significantly more frequent LOH in high grade tumors than in those of lower grades ($P < 0.05$).

Tumor stage had been recorded for 24 of the 41 (59%) high grade cancers in this study and for each of the 40 cases in the previous study. We also combined the two studies and correlated tumor stage with 13q LOH. At region one, LOH frequencies were 11% (3/28), 13% (2/15), and 57% (12/21) for tumors of stages II, III, and IV, respectively; and LOH frequency for stage IV was significantly higher than that for stage II ($P = 0.001$) or III ($P = 0.021$). At region two, the frequencies were 21% (6/28), 20% (3/15), and 43% (9/21) respectively; and no significant difference was seen between different stages ($P > 0.05$). When analyzed for having LOH at either region one or region two, tumors of stage IV again showed significantly more frequent LOH than did those of stage II ($P = 0.003$) or III ($P = 0.009$).

To determine whether LOH regions one and two were continuous from one to another or they were independent, we identified the cases (30 in total) with LOH at either region one or region two or both. While half of the 30 cases showed LOH at region one, region two, and the control marker between them, another half showed LOH at either region one only (8 cases), or region two only (5 cases), or showed LOH at both regions one and two but not at the control marker (i.e., D13S166) between them (2 cases) (Fig. 1).

DISCUSSION

In this study, we analyzed 41 prostate cancers obtained from transurethral resection (TUR) for LOH

at three previously identified LOH regions at 13q [14], i.e., regions one, two, and three at 13q14, 13q21, and 13q33, respectively. Compared to LOH frequency at "control" markers on 13q, each of the three regions showed more frequent LOH. Consistent with previous studies [8–15], these findings provide more evidence that chromosome 13 harbors multiple genes whose inactivation is involved in the development and/or progression of prostate cancer.

Compared to the previous study in which LOH frequencies were 5/39 (13%) at region one, 6/36 (17%) at region two, and 5/36 (14%) at region three in prostate cancers from prostatectomy specimens [14], LOH frequencies at regions one and two but not at region three were significantly more frequent in the cancers in the current study. This finding suggests that LOH at regions one and two is associated with clinically more significant prostate cancers, as the neoplasms used in this study produced local clinical symptoms (bladder outlet obstruction) while those from the previous study were detected by the PSA test. The neoplasms in the current study were predominantly high grade and stage IV tumors, while those in the previous one were mainly lower grade and stages II and III cancers. As patients with higher grade or stage prostate cancers have worse outcomes than those with lower grade or stage neoplasms [18], the frequent LOH at 13q is therefore associated with more advanced cancers. Consistently, tumors with stage IV showed significantly more frequent LOH at region one than those with stage II or III. Although the target genes have not been identified at any of the LOH regions at 13q, our findings suggest that deletions at these loci are associated with advanced features of prostate cancer.

Comparison of LOH frequencies according to tumor grade by combining the two studies revealed that the region at 13q14 showed an increased LOH frequency in high grade cancers (Gleason score 8–10) than in those of lower grade (score 5–7). The LOH region at 13q21 showed similar LOH frequencies between low and mid grade tumors and its LOH frequency was not significantly higher in high grade cancers compared to lower grade neoplasms (Fig. 3).

The region at 13q33 was lost less often than those at 13q14 and 13q21 in advanced prostate cancers. This is consistent with a previous study in which this locus had the least frequent LOH in metastases of prostate cancer [14]. These results suggest that the 13q33 locus may not be critically important in the progression of prostate cancer.

This study suggests that LOH at 13q14 and 13q21 does not represent the same genetic event. The LOH pattern according to tumor grade or stage was different between regions one and two (Fig. 3). In addition, half of the 30 cases with LOH at one or two of the two regions lost region one only or region two only or lost both regions but retained the control marker between them. These results indicate that the genetic deletion on 13q is not contiguous, and LOH at 13q14 and 13q21 are two independent events that may represent loss of two distinct but unknown genes. This is consistent with our previous study [14].

There are several known or candidate tumor suppressor genes that map to chromosome 13, i.e., *BRCA2* (13q12) [19], *RB1* (13q14) [20], *DBM* (deleted in B-cell malignancy) (13q14) [21,22], leukemia-associated genes 1 and 2 (13q14) [23], and the endothelin receptor B (*EDNRB*) (13q21) [24]. The 13q14 region deleted in prostate cancer is telomeric to *BRCA2* and centromeric to *RB1*, and neither of these known genes appears to be critically involved in prostate cancer [14]. The *DBM* and leukemia-associated genes 1 and 2 are further telomeric to the *RB1* gene [21–23]. At 13q21, the *EDNRB* gene is at least 3 cM telomeric to the common region of deletion in prostate cancer [25]. Currently there are no genes that are located in the regions of deletion that have been identified as tumor suppressor genes.

In summary, we found that LOH at 13q14 occurs more frequently in clinically significant prostate cancers and this locus likely contains a tumor suppressor gene important in more advanced prostate cancers.

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Mutations of *PTEN/MMAC1* in Primary Prostate Cancers from Chinese Patients[1]

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ABSTRACT

***PTEN/MMAC1* is a putative tumor suppressor gene located on 10q23, one of the most frequently deleted chromosomal regions in human prostate cancer. Although mutations of *PTEN* have often been detected in metastases of prostate cancer, localized tumors have shown lower rates of mutation, which have varied from 0 to 20% among different studies. It is unknown whether the rate of *PTEN* mutations is different in prostate cancer from Asian men compared with Western men. To further clarify the role of *PTEN* in prostate cancer and to examine the gene for mutations in Asian men, we analyzed 32 cases of primary prostate cancers from Chinese patients, each of whom was not diagnosed by screening with serum prostate-specific antigen, for *PTEN* mutations using the methods of tissue microdissection, single-strand conformational polymorphism, and direct DNA sequencing. Seventy % of the tumors were Gleason scores 8–10, whereas the remainder were Gleason score 7. Six metastases of prostate cancer from American patients were also analyzed. Five of 32 (16%) primary prostate cancers from Chinese men and two of six metastases from American men showed mutations in a total of 10 codons of *PTEN*, which involved exons 1, 2, 5, 8, and 9. Two of the mutations were truncation type, whereas the rest were missense mutations. The mutation frequency in these cases from Asian patients was higher than that in our previous study of cases in radical prostatectomy specimens from American men, in which the 40 primary tumors were lower grade and had been detected by serum prostate-specific antigen test. We conclude that mutation of *PTEN* occurs more often in primary prostate cancers of Chinese men, whose tumors are high grade and reflective of an unscreened population.**

INTRODUCTION

A candidate tumor suppressor gene designated *PTEN*, *MMAC1*, or *TEP-1* (referred to as *PTEN* hereafter) was identified (1–3) from the q23.3 region of chromosome 10, one of the most frequently deleted regions in prostate cancer (4). The *PTEN* gene has nine exons that encode a 403-amino acid protein of a dual-specific phosphatase with putative actin-binding and tyrosine phosphatase domains. Introduction of PTEN into cancer cells that lack *PTEN* function inhibits cell migration and induces cell cycle arrest and apoptosis via negative regulation of the phosphatidylinositol 3′-kinase/protein kinase B/Akt signaling pathway (5–7). Mutation and down-regulation of the *PTEN* gene have been detected in various human cancers including that of the prostate (8–10). In addition, germ-line mutations in *PTEN* are associated with Cowden disease (11), in which patients are at increased risk for certain cancers.

Thus far, *PTEN* appears to be the most frequently mutated gene in metastases of prostate cancer, occurring in at least 1 metastatic site in 12 of 19 (63%) patients who had multiple metastases (12) and in 9 of 15 (60%) cell lines and xenografts primarily derived from metastases of prostate cancer (13). These results indicate a role for *PTEN* in the progression of prostate cancer. Mutations of *PTEN* in localized prostate cancers have been found at lower frequencies including 1 of 28 (4%; Ref. 14), 1 of 25 (4%; Ref. 15), 1 of 40 (2.5%; Ref. 16), 0 of 45 (17), and 1 of 22 (5%; Ref. 18). Somewhat higher rates of mutations have been observed in other studies including 10 of 80 [12.5%; 10 of 23 (43%) in cases with loss of heterozygosity at *PTEN*; Ref. 19], 5 of 37 (13.5%; Ref. 20), 8 of 60 (13%; Ref. 21), and 1 of 10 (10%; Ref. 9). In hereditary prostate cancer, the role of *PTEN* has not been detected (22, 23).

The incidence of prostate cancer is lower in Asian men compared with Western men, but the specific genetic or environmental factors that are important are unknown (24, 25). Obviously, more cancers are detected in Western men because of screening with serum PSA[3] test. The frequency of PTEN mutations in prostate cancer from Asian men has been little studied. One study of 45 primary prostate cancers from Japanese patients did not detect any *PTEN* mutation (17). In this study, we analyzed primary prostate cancers from 32 Chinese patients, who were not diagnosed using the PSA test. Rather, they were diagnosed after showing clinical symptoms. We also analyzed six metastases from American patients who died of prostate cancer to document additional PTEN mutations in fatal prostatic disease.

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[3] The abbreviations used are: PSA, prostate-specific antigen; SSCP, single-strand conformational polymorphism.

MATERIALS AND METHODS

Tumor Samples. Thirty-two formalin-fixed, paraffin-embedded prostate cancer specimens from radical prostatectomy from previously untreated Chinese patients were used in this study. These patients went to physicians after showing various symptoms of prostate cancer, *e.g.,* difficulty in voiding, urodynia, urgent and frequent urination, and hematuria. None of them were involved in PSA screening. Their prostates were examined by one or more of the following means: rectal ultrasound detection, digital rectal examination, computed tomography, and magnetic resonance imaging. Biopsy was performed for the patients who were suspected to have prostate cancer, and only those whose cancers were at stages B–C underwent radical prostatectomy. The prostatectomies were performed by four surgeons over a period of 5 years. All specimens were from archived paraffin blocks that had been used in routine diagnosis of cancer, and none of them were collected specifically for this study. In addition, DNA was available from six distant metastases from American patients who died of prostate cancer. The clinicopathological characteristics of the tumors are listed in Table 1. The exact tumor stage for the Chinese patients was not available. Tumor cells for DNA isolation were collected from 7 μm H&E-stained sections by microdissection using a protocol described previously (26), which typically ensured a minimum of 70% neoplastic cells for each sample. Nonneoplastic cells collected were present on the same slides as cancer cells and included stromal cells, lymphocytes, and urothelium; in most cases, they did not include nonneoplastic prostatic epithelium. For the cases of metastases, nonneoplastic cells were collected from lymph nodes or seminal vesicles. Use of the human specimens in this study was approved by the institutional human investigation committee.

PCR-SSCP Analysis. Each of the primary prostate cancers was first screened for mutation by using the PCR-SSCP approach. Primers used for each *PTEN* exon were the same as described previously (16). PCRs for the SSCP contained 5–10 ng of genomic DNA, 1× PCR buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA], 1 μM of each primer, 3 μM of each deoxynucleotide triphosphate, 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 0.6 unit of Taq DNA polymerase, and 0.1 unit of Pfu DNA polymerase and was incubated at 95°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The ^{32}P-labeled PCR products were electrophoresed at 5 W overnight at room temperature in a 6% nondenaturing polyacrylamide gel in 1× TPE buffer [30 mM Tris, 20 mM PIPES (1,4-piperazinediethanesulfonic acid), and 1 mM Na_2EDTA (pH 6.8)] as described previously (27). PCR products were also analyzed in a 0.25× MDE gel (FMC BioProducts, Rockland, ME) containing 10% glycerol, which was also run at 5 W overnight at room temperature. After electrophoresis, the gels were dried and exposed to X-ray film for 1–2 days. Samples showing a bandshift for a specific exon were reamplified for both tumor DNA and matched nonneoplastic cells to confirm the shift, using the same conditions.

DNA Sequencing. For the samples which repeatedly showed a bandshift in the SSCP analysis, shifted bands were cut and immersed in 20 μl of H_2O, following the protocol described by Kukita *et al.* (27). Two μl of the released DNA were amplified by PCR using the same primers, as in SSCP analysis, in a 50-μl of reaction. The PCR conditions were the same except that 200 μM of each deoxynucleotide triphosphate and no [^{32}P]dCTP were used. These PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and were sequenced by using the ThermoSequenase Cycle Sequencing kit (USB) following the manufacturer's instructions. Sequencing data were collected and analyzed by using the ScanDNASIS and MacDNASIS software (Hitachi Software, San Bruno, CA).

For the six metastases of prostate cancer, which tended to be more homogeneous in neoplastic cells, their DNAs were amplified by PCR for each of the *PTEN* exons, and the resultant PCR products were purified and directly sequenced by the same procedure as described above. For an exon showing a mutation,

Table 1 Clinicopathological characteristics of prostate cancer specimens analyzed and mutation status of *PTEN* in each case

Case no.	Patient Age (yr)	Gleason score	*PTEN* mutation
80	NA[a]	7	No
82	52	9	No
83	64	8	No
84	51	10	No
85	NA	10	No
86	61	8	No
89	61	8	Exon 5 (T418C, polymorphism)
90	63	8	No
91	NA	9	Exon 2 (A1197G, R55G)
92	NA	8	Exon 5 (T302A, I101A; A403G, I135V)
95	60	10	No
96	65	8	No
98	NA	7	No
99	83	7	No
100	75	9	No
101	NA	9	No
102	NA	7	No
103	NA	9	No
104	75	9	No
105	74	9	No
107	83	NA	No
108	71	NA	No
109	NA	10	Exon 5 (A449G, Q150G)
110	65	9	No
111	70	7	No
113	67	7	Exon 1 (G58T, G20Stop)
114	59	8	Exon 8 (C814T, H272Y)
116	66	7	No
117	NA	8	Exon 9 (A1086G, polymorphism)
119	56	9	No
120	NA	7	No
121	66	7	No
42[b]	77	Lymph node	No
46[b]	75	Liver	No
47[b]	70	Lymph node	Exon 9 (A1031G, K344R; C1043T, T348I; A1144T, T382S)
48[b]	73	Lymph node	No
49[b]	75	Lymph node	Exon 5 (C328T, Q110Stop)
51[b]	66	Liver	No

[a] NA, not available.
[b] Lymph node and liver were the organ sites of metastases.

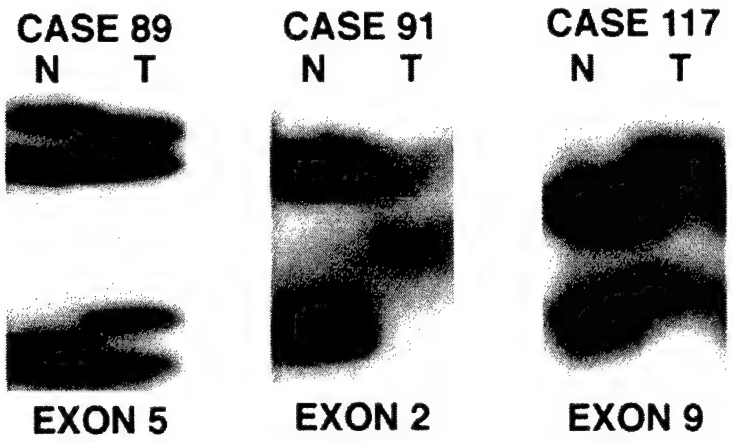

Fig. 1 Examples of SSCP analysis of *PTEN* in prostate cancer specimens. *N* and *T*, nonneoplastic and tumor cells, respectively. For each example, the case number is indicated at the *top*, and exon number is at the *bottom*. Each case has shifted bands in their tumor cells compared with nonneoplastic cells.

the PCR sequencing procedure was repeated to confirm the mutations. Once confirmed, matched normal DNA for a specific exon was also amplified by PCR and sequenced to determine whether a mutation was somatic or germ line.

Statistical Analysis. The difference in the frequency of *PTEN* mutations between primary tumors in the current study and that of our previous study (16) was analyzed statistically by the use of Fisher's exact test (two-tailed; Ref. 28).

RESULTS

Seventy % of the 32 primary prostate cancers from Chinese men were Gleason scores 8–10, whereas 30% were Gleason score 7. PCR-SSCP and direct DNA sequencing analyses of these samples revealed *PTEN* sequence alterations in 7 cases. Examples of bandshifts for tumors in SSCP assay, which indicated the existence of sequence alterations in the *PTEN* gene, are shown in Fig. 1, and examples of DNA sequencing ladders that identify *PTEN* mutations are shown in Fig. 2. Tumor cases and their *PTEN* mutation status are listed in Table 1. Although 2 of the 7 cases had alterations that did not change the PTEN polypeptide, five cases (16%) had mutations that could potentially change PTEN function (Table 1). Case 113 had a nonsense mutation at codon 20 that would truncate the majority of the PTEN protein. Case 92 had two missense mutations in its exon 5, which changed codons 101 and 135 from isoleucine to alanine and valine, respectively. Cases 91, 109, and 114 showed missense mutations that changed codons 55, 150, and 272 from arginine, glutamine, and histidine to glycine, glycine, and tyrosine, respectively.

We also analyzed six metastases of prostate cancer from American men, using the methods of PCR amplification and direct DNA sequencing. Two cases showed *PTEN* mutations. Case 49 had a nonsense mutation at codon 110 in exon 5 that would truncate the PTEN protein, and case 47 had three missense mutations in exon 9 of *PTEN*, changing codon 344 from lysine to arginine, codon 348 from threonine to isoleucine, and codon 382 from threonine to serine.

Each of the above mutations was somatic, as the matched nonneoplastic cells showed no mutations. The difference in the frequency of 16% for *PTEN* mutation in the cancers from Chinese patients compared with the frequency of 2.5% in our prior analysis of 40 resected primary tumors detected in American men after PSA test and biopsy (16) showed a trend in significance ($P = 0.08$).

DISCUSSION

The *PTEN* gene was isolated from the q23 region of chromosome 10, one of the most frequently deleted regions in prostate cancer (4, 29, 30). Mutations of the gene have been detected in various human cancers including that of the prostate (9, 12, 13, 19–21), implicating *PTEN* in the development and/or progression of prostate cancer. It is thus far the most frequently mutated gene in prostate cancer. Our finding of *PTEN* mutations in 5 of 32 primary, high-grade prostate cancer specimens confirms that *PTEN* is a major gene, if not the target gene, for the 10q23 region of deletion in a subset of prostate cancers.

Mutation frequencies of *PTEN* in prostate cancer differ among studies, largely because of differences in tumor grade and stage in the study populations. Mutations up to 60% have been detected in studies of prostate cancer cell lines and xenografts from metastases (13), whereas in some studies of localized disease, few or no mutations have been detected (16, 17). In this study, we detected *PTEN* mutations in 5 of 32 (16%) primary prostate cancers from Chinese patients who were diagnosed with clinical symptoms but without the aid of the serum PSA screening test. This frequency was higher than that (1 of 40 or 2.5%) detected in primary prostate cancers from American patients who were diagnosed by PSA test in our previous study (16). The majority of tumors from the Chinese patients were high grade (Gleason scores 8–10), whereas the majority of tumors in the American patients were lower grade (Gleason scores 5–7), indicating that PTEN mutations occur more often in tumors with high Gleason scores, even in those that are primary lesions. This conclusion is consistent with published studies of primary prostate cancers (15, 17, 20). In one study of 37 primary tumors with 20 (54%) high-grade and 17 (46%) lower grade lesions, five cases, four of which were high-grade tumors, had PTEN mutations (20). In another study of 45 primary tumors that were mainly low-grade cancers [30 (67%) lower grade cases and 15 (33%) high-grade cases], no PTEN mutations were

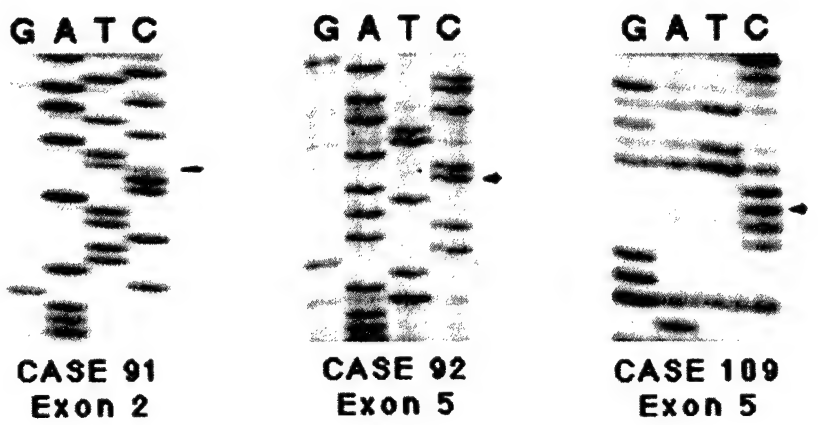

Fig. 2 Examples of sequencing analysis of shifted bands from SSCP experiments. *Arrow,* mutated nucleotide; *bottom,* case number and exon number.

found (17). Summarizing five studies in which both tumor grade and PTEN mutations were available (15–17, 20), we found that 9 of 67 (13.4%) high-grade tumors showed PTEN mutations, whereas only 3 of 117 (2.6%) lower grade cases showed mutations. The former rate is significantly higher than the latter ($P = 0.01$) using the χ^2 analysis-of-contingency table (28). Consistent with mutation studies, loss of PTEN expression has also been shown to correlate with high grade of primary prostate cancer (9, 10).

It has been reported that prostate cancer incidence is lower in Asian men than in Western men (24, 25). Although one study of Japanese patients did not detect any *PTEN* mutations in 45 primary tumors that were mainly low-grade cancers (17), we found more frequent *PTEN* mutations in a group of Chinese patients that had mainly high-grade tumors in this study; the latter is consistent with studies in Western men (20). These results suggest that *PTEN* is likely not a genetic factor contributing to the racial difference in prostate cancer incidence. This conclusion is further supported by the fact that all of the *PTEN* mutations were detected in prostate cancer cells only and not in their matched nonneoplastic cells. Also, no *PTEN* mutation has been detected in familial prostate cancers (22, 23). The differences in PTEN mutation rates in our study compared with that of Orikasa *et al.* (17) may be attributable to differences in the distribution of tumor grades between the study samples.

We detected multiple mutations for *PTEN* in two tumors, *i.e.,* case 92 had two missense mutations in exon 5 and case 47 had three missense mutations in exon 9 (Table 1). The heterogeneous nature of prostate cancer is well known (31); therefore, it is likely that multiple mutations of *PTEN* in one tumor may come from different subclones of tumor cells. In an analysis of metastases involving multiple organ sites in patients who died of prostate cancer, Suzuki *et al.* (12) found that different metastases within the same patient had different PTEN mutation status, indicating a complex genetic relationship between various subclonal lineages of prostate cancer cells. Mutation of exon 5 appears to be more frequent than that of other exons in both Cowden disease and various somatic cancers (8).

In summary, *PTEN* mutations were seen more often in primary prostate cancers from Chinese men compared with localized tumors from American patients. This difference is likely attributable to the presence of an excess of high-grade cancers in the Chinese patients. Whether primary prostate tumors with *PTEN* mutations have a greater proclivity to metastasize than those of similar grade and stage without mutations remains to be determined.

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GENES, CHROMOSOMES & CANCER 31:333–344 (2001)

Defining A Common Region of Deletion at 13q21 in Human Cancers

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Previous molecular genetic analyses identified a region of deletion at 13q21 in a variety of human cancers, suggesting the existence of a tumor suppressor gene(s) at this locus. In our earlier study on prostate cancer, the region of deletion was confined to a 3.1 cM interval between D13S152 and D13S162. At present, however, no known gene located in this interval has been firmly implicated in cancer, and the region remains too large for gene identification. To fine-map the area of interest, we established a contig of bacterial artificial chromosome (BAC) clones, narrowed the region of deletion by loss of heterozygosity (LOH) and homozygosity-mapping-of-deletion (HOMOD) analyses in different types of cancers, and tested a candidate gene from the region for mutation and alteration of expression in prostate cancers. The contig consisted of 75 overlapping BAC clones. In addition to the generation of 47 new sequence-tagged-site (STS) markers from the ends of BAC inserts, 76 known STS and expressed sequence tag markers were mapped to the contig (25 kb per marker on average). The minimal region of deletion was further defined to be about 700 kb between markers D13S791 and D13S166 by LOH analysis of 42 cases of prostate cancer, and by HOMOD analysis of eight prostate cancer cell lines/xenografts and 49 cell lines from cancers of the breast, ovary, endometrium, and cervix, using 18 microsatellite markers encompassing the deletion region. A gene that is homologous to the *WT1* tumor suppressor gene, *AP-2rep* (*KLF12*), was mapped in this region and was analyzed for its expression and genetic mutation. In addition to low levels of expression in both normal and neoplastic cells of the prostate, this gene did not have any mutations in a group of aggressive prostate cancers and cell lines/xenografts, as assessed by the methods of polymerase chain reaction-single strand conformational polymorphism analysis and direct sequencing. These studies suggest that a 700 kb interval at 13q21 harbors a tumor suppressor gene(s) that seems to be involved in multiple types of cancer, and that the *AP-2rep* gene is unlikely to be an important tumor suppressor gene in prostate cancer. The BAC contig and high-resolution physical map of the defined region of deletion should facilitate the cloning of a tumor suppressor gene(s) at 13q21.

INTRODUCTION

Deletion of a portion of the long arm of human chromosome 13 (13q) is a frequent event in human cancer. In addition to cytogenetically visible deletions (Pittman et al., 1987), a number of comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analyses have demonstrated a region of deletion at 13q21 (Visakorpi et al., 1995; Cher et al., 1996; Szymanska et al., 1996, 1998; Larramendy et al., 1997; Nishizaki et al., 1998; Nupponen et al., 1998a,b; Hyytinen et al., 1999; Knuutila et al., 1999; Mairal et al., 1999; Parente et al., 1999). In addition to prostate cancer, deletion at 13q21 is also detected frequently (the second most frequent chromosomal deletion, after 9p23–24) by CGH analyses in 73 different types of tumors (Knuutila et al., 1999), including cancers of the breast (Kainu et al., 2000; Larramendy et al., 2000), ovary (Arnold et al., 1996; Wasenius et al., 1997), and bladder (Kallioniemi et al., 1995), as well as glioblastoma (Kim et al., 1995). These studies suggest that a tumor suppressor gene(s) at 13q21 is inactivated during the development or progression of many different types of malignancy.

In our previous studies of deletion mapping in prostate cancer, we detected a distinct region of loss involving markers D13S269 and D13S162 at 13q21 (Hyytinen et al., 1999). In the prostate can-

Sequence data from this article have been deposited with the GenBank Data Library under Accession Numbers G67088-G67125, G67144-G67152, and AF312866–AF312872.

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cer cell line/xenograft LNCaP and PC-82, the common region of deletion was defined to a 3.1 cM area (Dong et al., 2000). In addition, we found that deletion at 13q21 was associated with aggressive prostate cancer, suggesting that an unknown tumor suppressor gene in this locus plays a role in prostate cancer progression (Dong et al., 2000). Two known tumor suppressor genes, *BRCA2* and *RB1*, are mapped to 13q12 and 13q14, respectively, and they are not located within the 13q21 region of deletion. A third gene, the endothelin receptor B (*EDNRB*) gene, is located at 13q21 and has been suggested to be a tumor suppressor in prostate cancer (Nelson et al., 1997, 1998). Based on our deletion mapping studies, however, *EDNRB* is located at least 3 cM telomeric to the common region of deletion (Dong et al., 2000). Using a previously described yeast artificial chromosome (YAC) contig as a framework, we screened two bacterial artificial chromosome (BAC) libraries (CITB and RPCI-11) by polymerase chain reaction (PCR) and database searching. A high-resolution BAC, sequence-tagged-site (STS), and expressed sequence tag (EST) physical map spanning nearly 2.5 Mb of genomic DNA from D13S152 to D13S162 was constructed by STS content analyses, EST mapping, chromosome walking, and BAC fingerprinting.

Recently, a *WT1/EGR1*- related zinc finger repressor gene, *AP-2rep* (*KLF12*), was cloned and mapped to the 13q21 region of deletion (Imhof et al., 1999; Roth et al., 2000). AP-2rep protein has been reported to down-regulate the expression of the AP-2 transcription factor in prostate cancer cell lines (Roth et al., 2000). These data suggest that *AP-2rep* could function as a tumor suppressor gene in prostate cancer. Therefore, we analyzed the *AP-2rep* gene as a candidate tumor suppressor gene for its expression and mutation in prostatic tissues. Only low levels of expression were detected in prostate tissues and cell lines, and no mutations were detected.

MATERIALS AND METHODS

Cancer Tissues and Cell Lines/Xenografts

Fifty-one pairs of matched zinc formalin-fixed, paraffin-embedded normal and cancerous prostate tissues were used in this study. Of these, 36 were primary tumors and 15 were metastases. Tumor cells were collected by the procedure of microdissection, and genomic DNAs were isolated as described previously (Hyytinen et al., 1999; Dong et al., 2000). The use of specimens had been approved by the University of Virginia Human Investigation Committee.

Four prostate cancer cell lines, i.e., LNCaP, DU-145, PC-3, and TSU-Pr1, and four xenografts, i.e., PC-82 (Dong et al., 2000), LAPC-3, LAPC-4, and LAPC-9 (Whang et al., 1998), were used in homozygosity-mapping-of-deletion (HOMOD) and mutation analyses. Forty-nine other human cancer cell lines were also used in the HOMOD analysis, which included 18 from breast cancer (i.e., MDA-Mb-436, MDA-Mb-415, MDA-Mb-231, MDA-Mb-175, MDA-Mb-157, MDA-Mb-468, MDA-Mb-453, MDA330, MDA134, BT549, BT474, MCF-7, T47D, HBL100, SK-BR-3, ZR-75-30, HS578T, DU-4475), 12 from ovarian cancer (i.e., OVCAR-9, SKOV-8, OVERC-420, ES-2, PA-1, GRATCH, 2774, SW626, SKOV3, CAOV3, CAOV4, BG-1), nine from endometrial cancer (i.e., RL95-2, HHUA, AN3CA, HOUA-C, HEC-59A, HEC-1-A, HEC-1-B, KLE, MESSA), and 10 from cervical cancer (i.e., CASK1, SIHA, C4I, C4II, ME180, C33A, HT3, MS751, HELA, Ish II).

DNA Markers

Eleven STS markers between D13S152 and D13S162 were used for the initial screening of the CITB human BAC DNA pools (Research Genetics, Huntsville, AL). Selection of markers was based upon the latest version of the integrated human genome map that is available on-line from the MIT/Whitehead Institute for Biomedical Research (http://www-genome.wi.mit.edu) (Hudson et al., 1995). Forty-seven new STS markers were designed from the terminal sequences of BAC clones. Seventy-two ESTs were selected from the current Gene Map (http://www.ncbi.nlm.nih.gov/genemap/map.cgi?CHR=13) (Schuler et al., 1996) between anchor markers D13S1260 and D13S160. Twenty-two other STS markers were chosen from radiation hybrid G3 maps constructed by Stanford University (http://www-shgc.stanford.edu/Mapping/rh) or YAC-cosmid-STS maps constructed by the Columbia University Genome Center (http://genome1.ccc.columbia.edu/~genome/) (Cayanis et al., 1998). Primer sequences of these markers except for those generated in this study are available from the Genome Database (http://gdbwww.gdb.org). PCR primers were either purchased from Research Genetics or synthesized by Life Technologies (Gaithersburg, MD).

Polymerase Chain Reaction (PCR) and Reverse Transcription (RT)-PCR

PCR was performed in a volume of 10 μl, containing 1–2 μl of template DNA; 1× PCR buffer

TABLE 1. New STS Markers Developed From BAC End Sequences

Name	Forward primer	Reverse primer	AT[a] (°C)	Size (bp)	Accession ID
530F15-R	GAGTCCTAAATCTCTTCCTG	TCACTCTGGGCCTTAATG	57	131	G67088
R-31C6-F	CCCATACCTGCTAACTCACC	CATGTCTGCCGACCCTTC	60	132	G67089
R-31C6-R	TCAGAAAGTCAGGAGTAGGC	CTGGAAGATTGTTCTCACCC	60	133	G67145
497M21-F	GCAATTTTGGACTCCTTTAT	GCGGAAGTATTTCAGGCAG	60	164	G67090
497M21-R	TCCTGGCACATACTTGCAC	TATCCCTCCACAGTGTGTTC	60	149	G67091
220D4-F	GCAAGTTTACGCAGGATACG	ACATCCTTGCCAAACCTTG	60	208	G67092
220D4-R	GCTGGTGGCCCAGTAGTTC	GCTGGCATACATGTGAGCC	60	100	G67093
129N5-R	AATGCTTCTTGAAAGAGTGG	TTGCTCCTGAATCAAATGTC	57	167	G67094
R-33P2-F	TCACTGTTCATTGCCCTTAC	ATTGTTGGACACAGGCTATC	57	296	G67095
311L15-F	CCATAAACTGGGATTTCTCC	AGGCCATCAATGTCTCTGAG	57	130	G67096
311L15-R	GATGAAAGTCACCAGCAATC	GGCCCACATATCCTAACAAC	57	200	G67097
460E6-F	ATGCAGAAGAGTGAATCTGG	CACTGAATACACAGACCTGG	57	117	G67098
460E6-R	CTTCATTAGGTATTGGAGCA	AGGCACCCTAGAAGCTAGAG	57	213	G67099
348B20-F	GCAGGCATGCAAGCTTCTAC	CCCTTACCTCCCTTGGTTTCC	57	100	G67100
348B20-R	GCCAAGCAGAAAGGGTTATTCC	AGCTGGCCTCCATTAATTCT	57	143	G67146
292E14-F	GCAAGCTTCTCAGGGATTCTCA	CCTTGTCATTTTCCCCAACTCT	57	114	G67101
292E14-R	GAGATCCAAATCGAGTTAGGCA	GAAAACTCATTCTTTGGCAGCA	57	96	G67102
365O20-F	GAATAACACTCTCACGTCCC	TAATGCCCTCAGCTCATCC	57	143	G67147
365O20-R	GGAGGTATGGCTGGAGTGCT	CTGTGGTGAGGGTTAAATGG	57	106	G67148
R-108K20-F	TTGCTACTCTCATGGTCTAC	GCAGCAGTACAGCAGTAAG	55	117	G67103
R-108K20-R	AGTCAGGAAACGCAGCATC	TGTCAAAGTAATCCTTTTGCC	57	111	G67104
361B5-F	TCTATTCTTCAAATGTTGGG	CTAACTGGATCAATCACTGC	57	123	G67105
361B5-R	GGCCAGCAGCATTAGTTACC	GTATTGGCACTGCTGTGCAG	57	172	G67106
R-441J10-F	AAGCTAATGACCATTATCCC	CCATCATCACACCTCTCCTG	55	304	G67107
R-441J10-R	CCCACAGTTCCACAGTACC	CTACTGGACTTCTCCGAACC	55	170	G67108
R-1E18-R	TCCCCAAGCCTCTAGCTCTG	CTTTCTACGTCACAGGCTGG	55	165	G67109
233F8-F	TCAGGGATCTGAAAGCAAGC	GCTGCATCCAGTGTTAGCTC	57	167	G67110
233F8-R	CTAAACCCATCCAACTCAAG	CTACCCGTCTGCTTTGCAC	57	177	G67144
217B14-F	GGAGAGTGTAAGAATCTGCC	GCAATGGTGATCTAAGAGTC	57	126	G67111
217B14-R	CTACCAGCACATCACTGGC	GAGCTATCATTTCACTAGATGG	57	105	G67112
387O13-F	CTCTCGTTCTTTCATAGGGG	CAACTTTCCTGACAACCGC	55	109	G67113
352D6-F	AGTTACTCACCCTTTGCCAG	GATGACCTTCCCTGTAACGC	55	179	G67114
352D6-R	TTCCAGTCCATTAAGACACG	TTTGATGCAGCGGTTATGTA	55	139	G67149
440F8-F	GCGTAAGCAAGTGTTTTCTC	ACATTGGGATGGGGAGTTC	55	160	G67115
440F8-R	CCACGGCAGGACTGTTTCAG	TGGATTCAAAAGCCTCTGGC	55	130	G67116
571B18-F	CTTAGCCTATCAATTTGCAG	GTTTGGACCATAACCTATCC	57	99	G67117
571B18-R	GAACTTGTATTCAGCCCTGG	CTATGGAAGGATAGGCTGCC	57	202	G67118
R-679B14-R	TCTGGGTAGAGCATTCCGAG	CAATCCCACTCCTCTTCGTG	55	149	G67119
R-125B8-F	CTAAACCCCTTGATTCTGCC	AACCCTGTCTGCTCCAAGGT	55	195	G67120
R-125B8-R	GGTTCCTGCTGGTAAGAATG	CATCCTCCTGAATGTCACTG	55	176	G67121
366M18-R	TAGGTATCGTTTTGCCAGCCCA	GCTGACACTGGCTGTTGAAAGG	57	99	G67122
341M12-F	CATCCCATTCTGGAACTTGTCC	TGGGTTTGCATCTACTTCTGGC	57	119	G67150
341M12-R	GACAAGGGATTGCACAAGATTG	GCCACTGGCTCAATTTCTTACC	57	76	G67123
437C14-F	CTGAAGGCGAGATCTTACCTGC	TGAGGGCAGGGATCAATTCTTA	57	113	G67151
437C14-R	TCCATCGTCTAATATGCTGTG	GGAGAAGGGGAGTATGTAGAAC	57	101	G67152
194I5-F	TATTCATCCAGCCCCCTCAATG	AGATGTGCAGGAGATGAATGGC	57	95	G67124
194I5-R	ATTGTGGAGCAGTAAGAATTTC	AGCTGTCTGTAGTGAGGAATCA	57	96	G67125

[a]AT, annealing temperature.

(20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA); 0.4 μM of each primer; 200 μM of each dNTP; and 0.3 U of Taq DNA polymerase. After an initial denaturation at 95°C for 5 min, 30–35 cycles, each consisting of denaturation at 94°C for 15 sec, annealing at 55–57°C for 15 sec, and extension at 72°C for 30 sec, were performed in a GeneAmp 9600 thermo-cycler (Perkin-Elmer, Norwalk, CT). Total RNA from normal human prostates was purchased from Clontech (Palo Alto, CA) and was treated with DNase I to remove residual genomic DNA. The treated RNA was transcribed into cDNA using the Superscript II RNase H^- Reverse Transcriptase (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol.

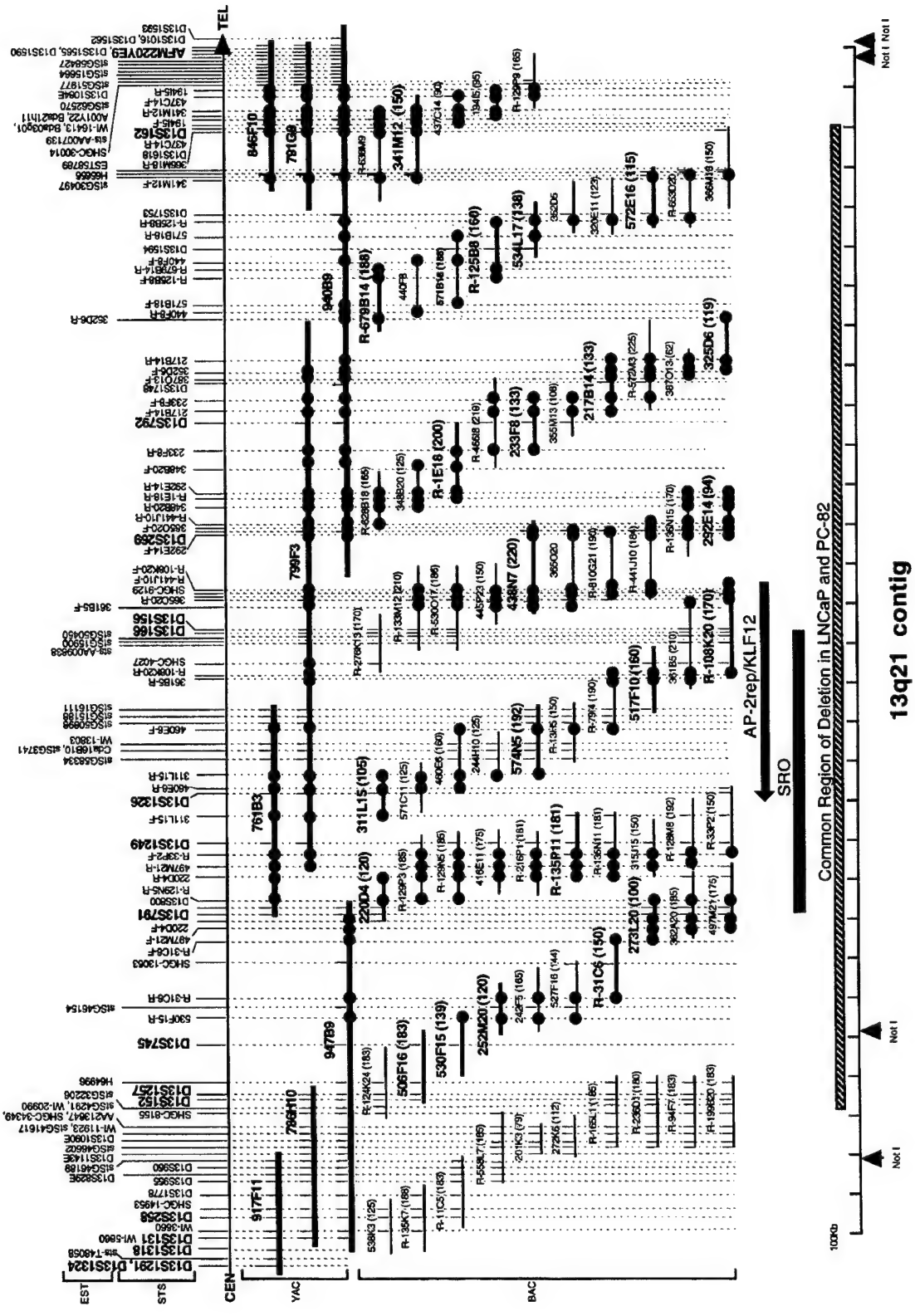

TEL
CEN
EST
STS
YAC
BAC
AP-2rep/KLF12
SRO
Common Region of Deletion in LNCaP and PC-82
13q21 contig
Not I
Not I Not I
100Kb

Figure 1.

YAC DNA Isolation

YAC DNA was isolated by a rapid method as described previously (Birren et al., 1997).

BAC Library Screening

Forty-one human BAC clones were identified by PCR-based screening of the human CITB BAC libraries (Research Genetics). Isotope α-^{33}P-dATP (3,000 Ci/mmol, ICN, Irvine, CA) was added to PCR tubes, and the radioactive PCR products were separated in 5% non-denaturing polyacrylamide gels (20 × 20 cm in size) at 220 V for 1 hr. The gels were dried and exposed to Kodak Biomax MR films at room temperature overnight. Thirty-four other BAC clones from the human RPCI-11 BAC library had been previously assigned to this region by PCR, fluorescence in situ hybridization (FISH), or fingerprint analyses by the Cancer Chromosome Aberration Project and were identified by searching the database at http://genomics.roswellpark.org/.

Determination of DNA Sequences for BAC Insert Ends

A 100 ml culture of a BAC clone was prepared for DNA isolation by using the Wizard plus Midipreps DNA purification system (Promega, Madison, WI), following the manufacturer's protocol. Ten to 20 μg of DNA were sequenced using T7 and SP6 primers, respectively, with an ABI 377 sequencing unit. The sequences obtained were tested using both the BLAST (http://www3.ncbi.nlm.nih.gov/BLAST/) and RepeatMasker (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker) programs to rule out repetitive sequences. Unique sequences were selected for designing primer pairs (Table 1). Several BAC-end sequences from the RPCI-11 library were obtained from The Institute for Genome Research human BAC ends database (http://www.tigr.org/).

Estimation of BAC Insert Size

DNA was isolated from a 3 ml culture of a BAC clone using the alkaline lysis procedure and then digested with the restriction endonuclease *Not*I (New England Biolab, Beverly, MA), that released the insert of the BAC clone. Digested DNA samples were loaded onto 1% agarose gels and separated by electrophoresis in 0.5 × TBE for 16 hr, using a Hoefer PC500 Pulse Controller (Pharmacia, Piscataway, NJ) at 200 V with a pulse time of 1–15 sec. Gels were stained with ethidium bromide, and DNA was detected by a UV transilluminator. The sizes of inserts were determined by comparison with size standards composed of a λ ladder (Bio-Rad, Hercules, CA).

Restriction Fingerprinting of BAC Clones

BAC clones were fingerprinted as previously described (Birren et al., 1997). Briefly, BAC DNA was digested with restriction endonuclease *Hin*dIII and separated in 1% agarose gels by electrophoresis in 0.5 × TBE for 16 hr at 200 V, in a field inverted with a pulse time of 50 msec. Gels were stained with ethidium bromide and detected by UV. Fingerprints for some BAC clones from the RPCI-11 library were downloaded from the Genome Sequence Center at Washington University (http://genome.wustl.edu/gsc/human/human_database.shtml).

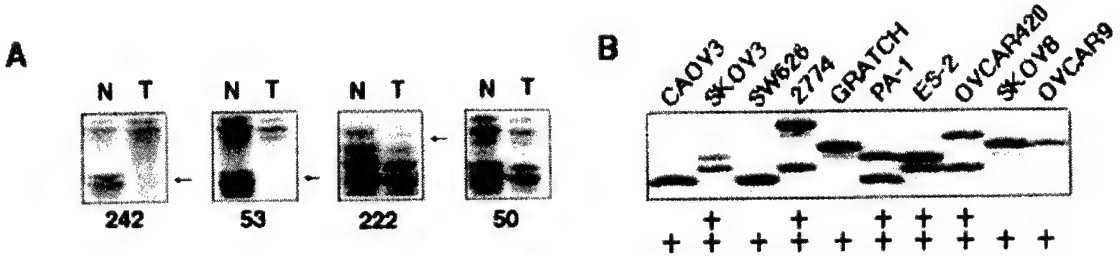
LOH and HOMOD Analyses

Eleven well-mapped microsatellite markers were used for LOH analysis as described previously (Hyytinen et al., 1999) (Figs. 1 and 2). The average distance between these markers was 300 kb. Eighteen microsatellite markers were used for HOMOD analysis (Goldberg et al., 2000) (Figs. 1 and 2), in which deletion is defined when more than five consecutive polymorphic markers with a heterozygosity of at least 0.75 show a homozygous genotype. PCR was carried out as previously described (Hyytinen et al., 1999). PCR products were separated in 6% denaturing polyacrylamide gels and detected by autoradiography. LOH was determined when the signal for one allele in the tumor was reduced significantly as compared with that for the nonneoplastic cells. All results were reviewed independently by two investigators (C.C. and J.-T.D.).

PCR Primers for Mutation Screening of the *AP-2rep* Gene

Although the genomic structure of the human *AP-2rep* gene was available (Roth et al., 2000), the adjacent intron sequence was not published. Based on the physical map constructed in this study, the *AP-2rep* gene was identified in CITB BAC clones 574N5 and 517F10. We designed a number of primers from the exon sequences to determine the exon/intron boundaries using DNA from these two

Figure 1. High-resolution YAC/BAC/STS/EST physical map for the deletion region at 13q21 in human cancers. EST and STS markers are listed on the top, and YAC and BAC clones are indicated by thicker and thinner horizontal bars, respectively. The names and sizes (kb) of YAC and BAC clones are listed above the bars. DNA markers in bold are microsatellite markers. Dots on YAC/BAC lines represent new STS markers generated from BAC terminal sequences. All BAC clones in this contig are drawn to scale. YAC clones outside the BAC contig are not drawn to scale. BAC clones depicted by thicker lines form the tiling path. *Not*I sites are based on BAC digestion experiments.



C

Markers	Prostate Tumors										Cell Lines				
	43M1	53M	79T	108T	105T	242T	222T	78T	50P	49P	HOUA-C	HELA	MDA330	LNCaP	PC-82
D13S 1291 (0.60)															
D13S 1318 (0.79)															
D13S 131 (0.86)															
D13S 258 (0.88)															
D13S 152 (0.78)															
D13S 745 (0.49)*															
D13S 791 (0.78)*															
D13S 1249 (0.46)															
D13S 1326 (0.69)															
D13S 156 (0.81)															
D13S 166 (0.76)															
D13S 269 (0.71)															
D13S 792 (0.77)*															
D13S 162 (0.86)															
AFM220YE9 (0.59)*															
AFM350XA5 (0.61)*															
D13S782 (0.75)*															
D13S160 (0.81)															

LOH MSI Heterozygosity Homozygosity Data not available

Figure 2. Deletions at 13q21 in prostate tumors and cell lines from different types of cancers. **A:** Examples of LOH at locus D13S162 in prostate tumors. Allelic status for normal cells (N) and matched tumors (T) are shown for four individuals. Case numbers are shown below each panel, and deleted alleles are indicated by arrows. There is no deletion in Case 50. **B:** HOMOD examples for eight ovarian cancer cell lines at D13S791. Double allele is denoted by "++", and single allele is denoted by "+". Abbreviated cell line designations are provided at the top of the panel. **C:** Allelic losses identified in prostate tumors and cancer cell lines using microsatellite markers. For prostate cancer tumors, only those samples with LOH are shown. For the cell lines, the 21 cell lines showing extensive losses that span the entire region of 13q21 are not shown. The order of these markers is from centromere to telomere. Heterozygosity values for markers are provided in parentheses. The heterozygosity values for some markers (marked with stars) were not available in the GDB and were estimated from cell lines without deletions in this study. Tumor cases and cell line names are listed at the top. Markers defining SRO for deletion are shaded. T, primary tumor; P, primary tumor from patients who had metastasis (M); MSI, microsatellite instability.

BAC clones. PCR primers were designed from the intron sequences to amplify all seven exons. Five pairs of primers were designed for exon 3 due to its larger size. Primer sequences, annealing temperatures, and PCR product sizes for the *AP-2rep* gene are listed in Table 2.

PCR-Single Strand Conformation Polymorphism (SSCP) Analysis and Sequence Determination

Each DNA sample was amplified by PCR in two rounds. The first-round PCR was performed as described above in the section of PCR, except that each sample was amplified in dual reactions, each

TABLE 2. Primer Sequences, Annealing Temperatures, and PCR Product Sizes for PCR Amplification of the Human *AP-2rep* gene

Exon no.	Primer sequences		AT[a] (°C)	Size (bp)	Accession ID
1	GGATCAATGTGACTCTA	CATCTGACTAACCAAC	55	81	AF312866
2	GAATGCTTTAGAGCTGAATC	CTCTCATCAAAAGGAGTGG	57	177	AF312867
3–1	AGTGGTAACCCATGCATCG	GGCTCCCCTTTCACATTATT	57	144	AF312868
3–2	TATGGAAGCCGTTCCCCTG	GTTGAAGAAGGTGAGGAGGC	57	189	AF312868
3–3	TCCCCAGTTTCCATGACAG	GTACGGGATGGATAATGTGC	57	208	AF312868
3–4	GCCTCTGCATCTGGTGTTGG	GCGGCACGACAATAGTGTTG	60	193	AF312868
3–5	GTCTAACAAACTGAGTCATGT	GAAGTCACCTCTAGTGCTG	57	193	AF312868
4	CTGCGCACTGATTTTAATTC	TTCCATTATCCATTGAACAC	57	241	AF312869
5	CTGGATTATTCTTCTCTGTCT	AGCCCTATTAAAGTTTCCC	57	147	AF312870
6	ATGGGGCGGGTGTGCATC	CTCACTGGCTCACAGGTGAGAA	60	247	AF312871
7	AGCACCAAAGCTTGTGTTCC	GAGATCCAGCTCTTACGCTC	57	256	AF312872

[a]AT, annealing temperature.

included all the primers for the seven exons, and Taq antibody (Life Technologies) was added. After 35 cycles of amplification, the PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and were eluted with 100 μl H_2O. One microliter of the diluted PCR products was used as the template for the second-round PCR, which was carried out under the same conditions as in the first round except that only a pair of specific primers, 1 mM of each dNTP, and 1 μCi α-^{33}P-dATP were used and 30 cycles were performed. The labeled PCR products were diluted 10-fold with denaturing buffer, heat-denatured, and electrophoresed at 40 W in a 6% non-denaturing polyacrylamide gel. The TME gel running buffer, which has a lower pH than TBE buffer, thus enhances the detection of band shifts (Kukita et al., 1997), and two temperatures (25°C and 4°C) were used for each sample. After electrophoresis, the gels were dried and exposed to Kodak Biomax MR film at room temperature overnight. Only the samples whose duplicates showed the same band shift were considered sequence alterations. The samples showing band shifts were re-amplified by PCR in 50 μl, and the DNA was purified with the QIAquick PCR purification kit and sequenced with an ABI 377 sequencing unit.

RESULTS

YAC/BAC/STS Physical Map

To identify the YAC clones that span the deletion region at 13q21, we examined STS markers between D13S152 and D13S162, which defined the region of deletion in our previous study (Dong et al., 2000), in the human genomic map database at the MIT/Whitehead Institute for Biomedical Research (Hudson et al., 1995) and the high-resolution YAC-STS-cosmid map of human chromosome 13 (Cayanis et al., 1998). All YACs from these two databases were cross-checked, and only those that contained at least three known STS markers at 13q21 were selected to minimize the number of possible chimeric clones. Eight human YAC clones were chosen and assembled into a contig (Fig. 1). Using 11 published STS markers that had been mapped within the region, overlaps between these YACs were confirmed by PCR-based STS content mapping. This YAC contig map was used as a framework for constructing the BAC contig.

Initial screening of the CITB BAC library with 11 STS markers generated at least one BAC clone for each marker. Additional BAC clones for the deletion region were obtained from the BAC contigs that had been established from the human RPCI-11 BAC library by FISH mapping and fingerprint analyses. Overlaps between these BAC clones were established and confirmed by PCR-based STS content analyses. For those BAC clones that did not have overlaps with other clones, their insert ends were subjected to direct DNA sequencing, and PCR primers were designed from these sequences to generate new STS markers, which were used to establish additional overlaps between BAC clones. If no overlaps were found, these new STS markers were used to screen the CITB BAC library to identify new BAC clones. Chromosome walking was continued until all gaps were filled.

To identify expressed sequences encoded by the 13q21 BAC contig, we searched the current Human Gene Map (Schuler et al., 1996) and selected 72 ESTs from the region between D13S1260 and D13S160, which covered the region of deletion at 13q21. After PCR amplification of these ESTs in YAC and BAC clones, 24 were mapped to both the

YAC and BAC contigs, and an additional nine were mapped to the YAC contig alone. Full-size genes (UniGene Hs.179566, *PIBF1*, *KLF5* (*BTEB2*), *KIAA1008*, *AP2-rep*, and *KIAA0603*) were available for six of these ESTs, based on a search of the GenBank database. Other ESTs were not detected in either the YAC or the BAC contig, which included two candidate tumor suppressor genes (*protocadherin 9* or *PCDH9* and *EDNRB*). In addition, four ESTs from the 13q high-resolution map (Cayanis et al., 1998) (D13S829E, D13S1143E, D13S1090E, and D13S1084E) were placed in the BAC contig by EST content mapping. Another two ESTs (361B5-F and 352D6-R) were identified by BLAST analysis of the EST database using DNA sequences generated from BAC insert ends (BACs 361B5 and 352D6). It was known that some EST clusters were from the same gene. These ESTs were evaluated for expression in a normal prostate using the method of RT-PCR. By comparing to β-actin's expression level, which is high in most cells, four ESTs (stSG46189, D13S829E, WI-20990, and stSG46154) showed a relatively high level of expression, whereas five had medium levels and 10 showed low levels of expression. The EST results are summarized in Table 3.

The high-resolution YAC/BAC/STS/EST physical map (Fig. 1) includes 123 markers (41 ESTs and 82 STSs); 47 of the STS markers were developed from the BAC contig (Table 1). A total of 75 BAC clones from two libraries were identified and assembled into the contig. The average insert size for the BAC clones was 156 kb, as estimated by *Not*I digestion and separation by inverted-field gel electrophoresis. The extent of overlap between BAC clones was determined by STS-content analysis and *Hin*dIII fingerprinting. The entire BAC contig spanned about 3 Mb and covers the common region of deletion defined in LNCaP and PC-82 prostate cancer cells. The tiling path from D13S152 to D13S162 is comprised of 22 BACs, and the size of deletion region is about 2.5 Mb (Fig. 1). The coverage of BAC clones for the region is 4-fold. The average marker resolution in this map is 25 kb per marker.

LOH and HOMOD Analysis

To narrow the smallest region of overlap (SRO), we analyzed 11 microsatellite markers that had been well mapped in the BAC contig (Fig. 1) in 42 human prostate tumors. Ten of the 42 cases (24%) showed LOH in this region. Whereas two metastases and three primary tumors showed extensive LOH, five cases showed regional LOH and narrowed down the SRO to 700 kb between D13S791 and D13S166 (Fig. 2).

HOMOD analysis was performed in 57 cell lines/xenografts from human cancers of the prostate, breast, ovary, endometrium, and cervix. Deletions at 13q21 were detected in 26 of the 57 (46%) cell lines, which included three of eight (38%) from prostate cancer (LNCaP, PC-82, LAPC-3), 12 of 18 (67%) from breast cancer (MDA-Mb-436, MDA-330, MDA-Mb-231, MDA-Mb-157, MDA-Mb-468, MDA-Mb-453, BT549, BT474, MCF-7, T47D, HBL100, HS578T), six of 12 (50%) from ovarian cancer (OVCAR-9, SKOV-8, GRATCH, SW626, CAOV3, BG-1), two of 10 (20%) from endometrial cancer (HOUA-C, KLE), and three of nine (33%) from cervical cancer (SIHA, MS751, HELA). Each of the three normal controls and 31 of the 57 (54%) cell lines showed no deletion. Twenty-one of the 26 cell lines with deletion had extensive losses at 13q21, as each of the markers showed homozygosity. The region of deletion with the lowest number of homozygous markers was found in the HOUA-C cell line, which consisted of 10 consecutive microsatellite markers and had a probability of 1.1×10^{-6} for a normal sample according to the heterozygosity value of each marker (Goldberg et al., 2000). In total, five cell lines showed partial deletions at 13q21 that defined the SRO for deletion to the same 700 kb between D13S791 to D13S166, as defined by LOH analysis in human tumors (Fig. 2).

Analysis of the *AP-2rep* Gene as a Candidate for the 13q21 Tumor Suppressor

Among the 10 ESTs located in the SRO for deletion, one gene, i.e., *AP-2rep*, had a full length cDNA available in the GenBank database. This gene spanned the SRO for deletion (Fig. 1) and is homologous to the *WT1* tumor suppressor gene. Therefore, we tested this gene as a candidate for the 13q21 tumor suppressor for expression and mutation in human prostate cancer cells. Repeated Northern blot analyses detected no signals of the *AP-2rep* transcript in normal prostate tissues and prostate cancer cells, whereas the control genes GAPDH and β-actin were detected with strong signals, indicating a low level of expression of *AP-2rep* (data not shown). RT-PCR analysis of *AP-2rep* showed that this gene was expressed in both normal prostate tissues and prostate cancer cell lines, but there was no obvious difference in the expression level between normal and cancer cells.

To determine whether *AP-2rep* is mutated in prostate cancer, we examined eight cell lines/xenografts derived from prostate cancer metastases, 13

TABLE 3. Transcripts Mapped to the Deletion Region at 13q21*

UniGene	Accession ID	Name	Known information	Expression in prostate
None	*T48058*	*Sts-T48058*	*EST*	−
None	AA233472	stSG46189	EST, NT2 neuronal precursor	+++
None		D13S829E	EST	+++
None	M78913	D13S1143E	EST	−
None		D13S1090E	EST	++
None	AA412034	stSG46602	EST	+
Hs.179566	U 79246	AA213647	Similar to enhancer of human tissue-type plasminogen activator	
	U 79246	SHGC-34349		
	U 79246	stSG4291		
	U 79246	WI-20990		+++
Hs.21738	AB023225	WI-11923	KIAA1008 protein	+
	AB023225	stSG41617		+
None	T85299	stSG32206	EST	−
Hs.252996	H64996	H64996	Moderately similar to PIBF1 protein	++
Hs.84728	AA213696	stSG46154	BTEB2/KLF5	+++
None	**W84891**	**stSG58334**	**EST**	**+**
Hs.169440	**Z39280**	**Cda16B10**	**EST**	**+**
	Z39280	**stSG3741**		
Hs.23510	**AJ243274**	**WI-13803**	**AP-2rep/KLF12**	**+**
	T56229	**stSG16111**		**+**
None	**AA028937**	**stSG58098**	**EST**	
None	**R08878**	**stSG15188**	**EST**	−
Hs.271776	**AA009838**	**sts-AA009838**	**EST**	+
None	**R92187**	**stSG15900**	**EST**	−
None		361B5-FE	EST	+
None	AA485229	stSG50450	EST	++
None		352D6-RE	SudD	−
None	AA167343	stSG30497	EST, torsin B (DQ1) mRNA	−
None	H65656	H65656	EST	+
Hs.173802	T33701	EST58789	KIAA0603 protein	++
Hs.60185	AA007139	sts-AA007139	EST	+
Hs.77917	G23955	SHGC-30014	Ubiquitin carboxyl-terminal esterase L3	++
Hs.5978	*Z24772*	*Bda03g01*	*LIM domain only 7, KIAA0858 protein, zinc finger domain containing protein*	
	G19783	*A001Y22*		
	Z28488	*Bda21h11*		
	G21209	*WI-16413*		
None	*N59881*	*stSG62570*	*EST*	
None	*AA425275*	*stSG51977*	*EST*	
None	*AA913382*	*stSG58427*	*EST*	
		stSG50944		
None	*R74041*	*stSG15664*	*EST*	
None		*D13S1084E*	*EST*	

*ESTs are listed according to the order from centromeric to telomeric. ESTs in italics are outside the BAC contig, but within the YAC contig. ESTs in bold are located in the SRO. Expression of ESTs in normal prostate was determined by RT-PCR. −, not detectable; +, ++, and +++ indicate low, medium, and high levels of expression, respectively. The expression of β-actin is defined as +++.

metastases of prostate cancer, and 20 primary prostate tumors, using the procedures of SSCP and direct DNA sequencing. The seven exons analyzed cover the entire coding region of the *AP-2rep* gene. These experiments revealed no tumor-specific band shifts, i.e., the *AP-2rep* gene is not mutated at a detectable frequency in prostate cancer. The SSCP analysis demonstrated two types of band shift, one for exon 2 and the other for exon 5, in both normal and cancer samples (Fig. 3A and 3B), indicating the existence of polymorphisms of the gene. In the 33 tumors for exon 2, 20 cases (61%) showed homozygosity for the smaller-band allele, six (18%) for the larger-band allele, and seven (21%) showed heterozygosity (Fig. 3A). For exon 5, 10 cases (31%) showed homozygosity for the smaller-band allele, 12 (36%) for the larger-band allele, and 11 (33%) showed heterozygosity (Fig. 3B).

Sequence analysis revealed that the polymorphism in exon 2 was in intron 1 at nucleotide 3

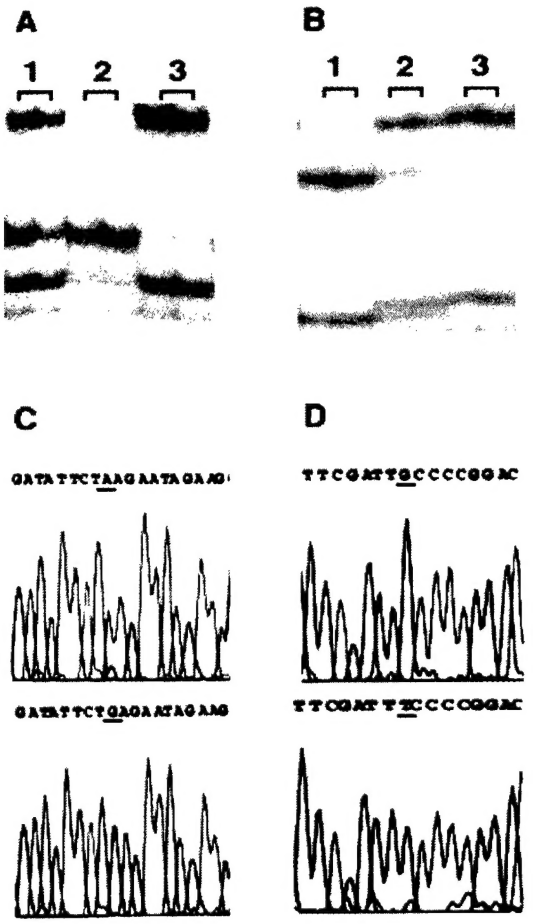


Figure 3. Detection of polymorphisms for exons 2 and 5 of the *AP-2rep* gene. **A:** Band patterns for three different genotypes at the polymorphism in exon 2. **Lane 1**, DU-145; **lane 2**, LNCaP; **lane 3**, patient 214. **B:** Band patterns for exon 5. Lane 1, PC-82; lane 2, PC-3; lane 3, DU-145. **C,D:** Sequences of polymorphisms for exons 2 and 5, respectively. In (C), the upper panel is from case 214 (lane 3 in A), and the lower panel is from DU-145 (lane 2 in A). In (D) the upper panel is from PC-82, and the lower panel is from DU-145. Nucleotide variations are underlined.

upstream to exon 2, with a thymine (T) in the allele showing a smaller band in SSCP analysis and a cytosine (C) showing a larger band (Fig. 3C). This polymorphism did not affect the amino acid sequence. For the exon 5 polymorphism, an A→C sequence change at nucleotide 840 (codon 280) was identified (Fig. 3D), with a C for the smaller-band allele and an A for the larger-band allele. This sequence alteration did not result in an amino acid substitution either.

DISCUSSION

We have constructed a physical map that integrates eight YAC clones, 75 BAC clones, 82 STS markers, and 41 EST markers for a 2.5 Mb common region of deletion at 13q21 shared by the LNCaP and PC-82 prostate cancer cell line/xenograft, as defined in our previous study (Dong et al., 2000). This map resolved the distances and relative orders for a number of STS/EST markers. For example, the human transcription factor dachshund gene (*DACH*) (Kozmik and Cvekl, 1999) was located between D13S152 and D13S162 by radiation hybrid mapping in the current Gene Map, but the BAC contig spanning D13S152 and D13S162 in this study did not contain the *DACH* gene. Similarly, four other ESTs, i.e., SGC33140, stSG31775, WIAF-541, and Cda0bd04, were not detected in the DNA interval between D13S152 and D13S162. In contrast, our BAC contig spanning D13S152 and D13S162 contained nine ESTs that were previously mapped either proximal to the region (i.e., STS-AA009838, stSG3741, stSG15900, stSG50898, stSG50450, WI-13803, stSG58334, and H64996) or distal to it (i.e., stSG30497).

The YAC/BAC/STS/EST map should be useful in fine mapping and eventual cloning of a new tumor suppressor gene at 13q21. We excluded a few candidate genes from further investigation, as they were not within the region of deletion. One such gene is *EDNRB* (EST D13162), which was found to be telomeric to the deletion region, consistent with our previous findings (Hyytinen et al., 1999; Dong et al., 2000). A zinc finger domain-containing gene, *KIAA0858*, was closer than the *EDNRB* gene to the deletion region, but is also telomeric. *PCDH9* is a new member of the cadherin superfamily and has been suggested to be a candidate suppressor gene (Strehl et al., 1998). Although the *PCDH9* gene was located between D13S1260 and D13S152 in the Gene Map, its two ESTs, A005R38 and A006D13, were not detected in the YAC/BAC contigs, suggesting that this gene is not located in the region of deletion (data not shown).

This high-resolution physical map has also been successfully used in this study to fine-map the region of deletion. Although previous studies identified a 3.1 cM region of deletion in prostate cancer, the size of the region with deletion was still too large for gene identification, and the precise location of the target gene remained to be elucidated. Using the polymorphic microsatellite markers mapped to the BAC contig, we performed loss of heterozygosity analysis in a number of human prostate cancer tissues and cell lines from different types of cancers to fine-map the region of deletion. As shown in Figures 1 and 2, these studies significantly narrowed down the SRO for deletion that is

now located between markers D13S791 and D13S166 in an interval of 700 kb.

According to the high-resolution physical map (Fig. 1), the SRO for deletion (700 kb) harbors the *AP-2rep* gene, which is homologous to the *WT1* tumor suppressor gene. AP-2rep protein contains a zinc finger domain and represses the expression of the AP-2 transcription factor in prostate cancer cell lines (Roth et al., 2000). It has been reported that AP-2 provides extra vital regulatory function for tumorigenicity of various types of cancer cells. For example, *NRAS*-induced transformation of human PA-1 cells resulted in a 6-fold elevation in *AP-2* mRNA levels (Buettner et al., 1993); over-expression of *AP-2* in breast cancer cells activated a set of target genes (i.e., the *ERBB2* proto-oncogene) that led to a hormone-independent, highly aggressive tumor phenotype (Bosher et al., 1995), and nuclear expression of *AP-2α* in prostate cancers correlated with a high Gleason score and was found to be an indicator of unfavorable disease outcome (Lipponen et al., 2000). Based on these findings, we examined the *AP-2rep* gene as a candidate suppressor gene for loss of function in prostate cancer. The expression level of this gene is quite low in normal prostate tissue, however, and no mutations of this gene were detected in a number of prostate cancer samples. Therefore, *AP-2rep* is unlikely to be the target tumor suppressor gene at 13q21.

In addition to prostate cancer cell lines and xenografts, cell lines from other cancers such as that of the breast and ovary also showed deletion at 13q21 (Fig. 2), suggesting that the same putative tumor suppressor gene is inactivated in different types of malignancies. This conclusion is supported by the findings from other analyses of breast and ovarian cancers (Arnold et al., 1996; Wasenius et al., 1997; Kainu et al., 2000; Larramendy et al., 2000), in which LOH and CGH studies frequently detected loss at 13q21. In addition, CGH analysis has demonstrated that sequence loss at 13q21 is the second most common deletion in 72 different types of tumors (Knuutila et al., 1999). In a recent study, a putative novel breast cancer susceptibility locus was linked to a region between D13S1317 and D13S166 (Kainu et al., 2000) that overlaps with the deletion region defined in this study.

In a recent study, HOMOD analysis was successfully used to detect LOH in cell lines, most of which do not have matched normal cells available for determining whether a homozygous marker indicates an allelic loss or noninformative status (Goldberg et al., 2000). In the HOMOD analysis, a region of deletion is defined if several consecutive polymorphic markers show single alleles in a cell line, and the probability for these markers to be homozygous in a normal individual is less than 0.001 (Goldberg et al., 2000). Our study further validated this procedure, as the regions of deletion detected in LNCaP and PC-82 prostate cancer cell line/xenograft in this study by HOMOD analysis exactly matched that defined by hemizygous deletion analysis in our previous study (Dong et al., 2000). Furthermore, both LOH and HOMOD methods defined the same SRO in this study. Therefore, HOMOD seems to be a reliable approach for the detection of LOH in established cell lines.

At present, there are eight other ESTs that are also mapped to the SRO, most of which are expressed in normal human prostate, as detected by RT-PCR analyses. With the completion of human genome sequencing for 13q21, more genes in this region of deletion will be identified. In summary, we constructed a high-resolution YAC/BAC/STS/EST map for a 2.5 Mb region that is frequently deleted in human cancers. The minimal region of deletion has been narrowed to a DNA segment of 700 kb between D13S791 and D13S166. Deletion of the same region was also detected in other major cancers. Finally, we excluded the *AP-2rep* gene as the 13q21 tumor suppressor gene in prostate cancer by testing its expression and mutation in a number of prostate specimens.

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